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EDITORIAL

Past Reflections and Future Vistas

As we step into the second year of publication, it is an appropriate time to take stock, reflect on the previous year and take a thick brush to paint the scenario of future plans and prospects. The recognition of the need for an international medium for rapid publication of communications in the nutritional sciences led to the genesis of *Nutrition Research*. The overwhelming response of the scientific community to the new Journal has been very satisfying. We set out to publish good quality papers in the shortest possible time. During the past year, the average time for review of manuscripts was 5 weeks and the maximum time elapsed between acceptance of an article and publication was 8 weeks. As might be expected, a number of papers had to be revised or turned down. Some individuals might query and find fault with the published material. However, all possible attempts were made to obtain peer review from two scientists in the field and then to arrive at a fair editorial decision. Peer review is still the most trusted and acceptable system of weeding out the less desirable material from the quality papers. It spares the reader much of the time and difficulty involved in assessing each manuscript. Much credit must be given to the anonymous reviewers who read the paper in full, refer to the appropriate literature and provide constructive comments usually based on the sincere desire to improve the quality of the presented data. In some cases, a fair amount of subediting has to be done, particularly in the case of papers received from countries where English is not the national language.

Nutrition Research initiated a number of new ideas. The main thrust of the publication is on *Original Articles*, both in human and animal nutrition. The rapidity of processing and printing ensures that important data reaches the readers within a few weeks of its compilation for publication. *Conference Summaries* highlight the important points made during workshops, congresses and other meetings, usually within 8-12 weeks of the conference. *Book Reviews* provide a critical analysis of recent monographs and edited volumes. This can help the reader to choose those publications which may merit an inspection prior to the final decision to purchase or to recommend for institutional library. *Editorial Comments* briefly highlight some of the material included in the Journal with reference to other appropriate literature. *Announcements* point to important events in terms of conferences planned, awards and fellowships available.

New features are planned for the future volumes. *Special Articles* will be included occasionally to publish material of unusual nature; one such manuscript described the unique data on protein-energy requirements from China. Quality *Review Articles* of high scholarship, intellectual concept, readability, accuracy and topicality will analyse current information on selected subjects. The size of literature explosion and information overload has led us to start new sections on *Brief Communications* and *Synopsis Article* with page limitations of 4 and 2 pages respectively. It is recognized that some manuscripts worthy of publication are turned down mainly because of intense pressure on space. We will encourage inclusion of the information contained in such manuscripts in a very

concise form, eliminating extensive literature review and discussion. Details of methodology, raw data and further information would be provided by the author(s) on specific request from readers. Finally, *Country and Region Reports* will provide material from countries where much of the scientific data are published in non-English journals or in publications with very limited distribution. By its inclusion in a summary fashion, such useful information will have an assured wider audience.

Nutrition Research will continue to strive to provide high quality scientific material in nutritional sciences and make it available to its readers in the minimum possible time. We are satisfied with the achievements of the past year but much more needs to be done. I wish to acknowledge my personal sincere gratitude to the Advisory Editorial Board members, Consultant Editors, reviewers of manuscripts and many other individuals whose help, advice and criticism is moulding the shape and contents of *Nutrition Research*. The publishing team, particularly Robert and Ian Maxwell, Robert Miranda, Phyllis Hall, James Alexander and Michael Sullivan have provided excellent support and cooperation. The chief credit, however, must go to the authors and readers whose efforts and response have given solid foundation to this Journal. I shall look forwards to your comments. Best wishes for the holidays and New Year !

St. John's, Newfoundland
January 1, 1982

Ranjit Kumar Chandra

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Food and Nutrient Intake of Infants During the First 18 Months of Life

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ABSTRACT

Food and nutrient intakes of normal infants, from 1 to 18 months, in Toronto and Montreal were determined in a longitudinal survey. The energy content of the diets, except for the first month, was less than the recommended daily intake (RDI). However, the infants were growing at normal rates, suggesting that the RDI is high. The mean intakes of most nutrients were consistently above RDI. Dietary iron was low from 12 months onwards, whereas, the intakes of Vitamin A, Vitamin C and sodium were high. Low iron intake was due mainly to low intake of iron-fortified infant cereals. Vitamin supplements, containing Vitamins A and C were largely unnecessary since food alone provided sufficient amounts to meet RDI. High sodium intake was related to consumption of salted table foods. Milk was the main source of energy, protein, fat and most other nutrients. Infant cereals, fortified with iron, calcium, phosphorous, thiamin, riboflavin, and niacin were important sources of these nutrients. Fruit and fruit juices were the primary sources of Vitamin C and vegetables the main sources of Vitamin A. Meat provided much of the dietary protein in the later infancy. The results stressed the importance of infant diets comprising the four basic food groups.

KEY WORDS: Nutrients, recommended daily intake, fortification, milk, infant cereals

The importance of adequate nutrition during infancy is without dispute. This early period in an individual's life encompasses rapid growth and development. As a result,it demands optimal intake of energy and essential nutrients (1).

As a means of assisting diet planners in providing optimal nutrition for the infant population, dietary standards were established (2). The standards recommend daily nutrient intakes (RDI) to meet the physiologic needs of the majority of the population. For this reason, the RDI are in excess of the minimum requirement of most individuals. Although the RDI are not designed for evaluation of adequacy of nutrient intakes of individuals, they, can be used to gauge how well infants eat.

The authors are indebted to Dr. G. H. Anderson for his expertise in the conduct of the survey. The H. J. Heinz Company of Canada Ltd acknowledges the support of an Industrial Research Assistance Program of the National Research Council of Canada.

In an attempt to determine how well Canadian infants were fed, a longitudinal survey of infant nutrition in the first 18 months of life was conducted (3). For this purpose, the type and quantity of food items consumed were determined. In this paper, the nutrient intakes of the infants are assessed against the Canadian Dietary Standard (Table 1). The primary sources of the nutrients are discussed. The objective is to identify problems in the diets with the aim that by revealing the problems, steps may be taken for their correction.

TABLE 1
Recommended Daily Intake of the Canadian Dietary Standard (2)

	0-6 months	7-11 months	1-3 years
Energy (kcal)	Body wt. (kg) x 117	Body wt. (kg) x 108	1400
Protein (g)	Body wt. (kg) x 2.2	Body wt. (kg) x 1.4	22
Vitamin A (RE)	400	400	400
Thiamin (mg.)	0.3	0.5	0.7
Riboflavin (mg.)	0.4	0.6	0.8
Niacin (NE)	5	6	9
Vitamin C (mg.)	20	20	20
Calcium (mg.)	500	500	500
Phosphorous (mg.)	250	400	500
Iron (mg.)	7	7	8
Sodium (mg.)	Body wt. (kg) x 30	Body wt. (kg) x 30	Body wt. (kg) x 30
Potassium (mg.)	Body wt. (kg) x 60	Body wt. (kg) x 60	Body wt. (kg) x 60

METHODS

A longitudinal nutrition survey was conducted in Toronto and Montreal, the two largest cities in Canada, between 1977 and 1979. Details of the survey and the demographic distribution of the sample are published elsewhere (3). The initial sample consisted of 403 women in late pregnancy. Sample distribution between the two cities was almost equal. The ratio of Anglophones to Francophones was 3 to 1 which was similar to the 1976 national census figures (4). At the start of the survey the procedure of the study was fully explained and written consent was received from the parents. All the infants were born within two weeks of the expected date of birth. The birth weight and length of the infants were within normal ranges (4). Thus the newborns were healthy normal infants.

The dietary intakes of the infants were determined by the 4-day dietary record method at 1, 3, 5, 6, 7, 8, 10, 12, 15, and 18 months. The 4-day dietary record method was selected as it was found, from 3 pretests, to provide optimum dietary information while maintaining co-operation from the parents. The parents were carefully instructed to keep accurate records of their children's food intakes at the stated times. All completed dietary records were scrutinized by our research dietitians. If necessary, the mothers were contacted for clarification or verification of information.

The number of subjects who submitted dietary records at each of the 10 study intervals were 403, 377, 362, 354, 330, 344, 335, 329, 321, and 317 respectively. The decreasing number of subjects was the result of attrition due to various reasons. For logistic reason, we could not determine breastmilk volume. Therefore, the total nutrient intakes of breastfed infants were not estimated. The data presented in this paper was obtained from the non-breastfed infants only. The respective sample sizes were 163, 226, 265, 279, 277, 302, 309, 314, 302, and 313. The increasing number of subjects at successive study intervals was the result of increasing number of subjects who

were weaned from the breast and included in the non-breastfed group.

The nutrient intakes were estimated using a modified version of the Nutrition Canada Nutrient Data Tape (5). At the start of the survey, it was updated with current food composition values. Data from commercial baby foods were derived from existing food composition tables. The nutrient contents of homemade foods were calculated from recipes provided by the parents. Where necessary, nutrient values were obtained by chemical determination in our food research laboratory.

The total food, energy and nutrient intake of each day and the average of the 4 days of each child were computer tabulated. The population mean, standard deviation and the mean expressed as percent of the RDI were also computer processed in the PL/1 language (6) at the University of Toronto. Data presented in this paper include: weight of foods consumed, energy, protein, fat, carbohydrate, calcium, phosphorous, iron, sodium, potassium, vitamin A, thiamin, riboflavin, niacin and ascorbic acid. Furthermore, contributions of the various food groups are also included.

RESULTS

The mean total quantity of food consumed by the infants increased rapidly during the first 6 months (Table 2). During the next 4 months, the increment was much slower. By 10 months, a plateau of about 1200 g. of food was reached. The large standard deviations showed the amount of food consumed varied widely.

TABLE 2
Food and Energy Intake of Non-Breastfed Infants

Age (months)	Food		Energy			Mean Energy Density (kcal./100 g.)
	Mean (g)	Sd.	Mean (kcal)	Sd.	% RDI	
1	764	177	502	121	102	66
3	876	165	597	116	86	68
5	1005	193	666	132	80	66
6	1080	208	717	141	80	66
7	1134	226	758	160	---*	67
8	1170	246	802	190	88	69
10	1212	245	872	202	89	71
12	1215	277	928	231	66	76
15	1216	291	1007	215	72	83
18	1219	302	1057	259	76	86

* Body weight of infants at 7 months was not determined

Milk was the primary source of food during the entire survey period. In the first 6 months, milk intake averaged 700 ml. In the proceeding months it gradually declined to 470 ml. at 18 months. Among the non-milk products, fruit and fruit juices were the main sources of food. This was followed by vegetables, meat, cereal products, dinners and desserts.

Total energy intake increased continuously even though the volume of food consumed plateaued at 10 months (Table 2). The energy density of food during the first 7 months was close to that of milk, which is 65 kcal/100 g., after which, it rose from 71 kcal/100 g. at 8 months to 86 kcal/100 g. at 18 months. Milk was the primary source of food energy. During the first year, cereal products and fruit juices were the secondary and tertiary sources. By the end of the first year, meat provided more food energy than either the cereal or fruit products. Except for the first month, the mean energy intake of the infants was less than RDI.

The protein and carbohydrate contents of the diets increased rapidly during the first 7 months, and at a slower rate in subsequent months (Table 3). The mean protein intake expressed as a unit of body weight was above RDI. Fat intake was stable, between 23 to 26 g. per day, during the first 8 months. A marked increase was noted from 8 months onwards. Throughout the 18 months, milk and meat were the primary and secondary sources of both protein and fat. In the first 7 months, milk was also the main source of carbohydrate. Beyond 7 months, fruit and fruit juices provided the most carbohydrate; milk and cereal products were the secondary sources.

TABLE 3
Protein, Fat and Carbohydrate in Diets of Non-Breastfed Infants

Age (months)	Protein			Fat		Carbohydrate	
	Mean	Sd.	% RDI	Mean	Sd.	Mean	Sd.
	(g)			(g)		(g)	
1	12.6	5.2	137	25.6	7.2	54.6	15.2
3	16.3	8.2	137	25.9	6.7	74.2	20.3
5	24.0	10.6	169	24.3	7.3	87.4	24.5
6	28.5	10.8	185	24.2	7.9	95.9	25.9
7	33.0	10.9	---*	23.4	7.8	103.5	29.5
8	35.1	10.9	298	24.2	8.8	111.0	32.9
10	38.3	11.4	303	27.2	10.2	119.2	32.1
12	40.9	12.4	186	30.5	12.0	123.5	34.5
15	42.8	12.0	195	35.7	11.7	130.9	33.7
18	44.3	14.3	202	37.7	13.6	138.1	38.5

* Weight of the infants at 7 months was not determined

Proportionally, carbohydrate provided the greatest amount of dietary energy (Table 4), with fat as the secondary and protein the tertiary sources. From 7 to 12 months, fat supplied less than 30% of the total dietary energy.

TABLE 4
Percent Energy Contributed by Protein, Fat and Carbohydrate in diets of Non-Breastfed Infants

Age (months)	Protein	Fat	Carbohydrate
	% dietary energy		
1	10	46	44
3	11	39	50
5	14	33	53
6	16	30	54
7	17	28	55
8	18	27	55
10	18	28	54
12	18	29	53
15	17	32	51
18	16	32	52

The mean calcium and phosphorous intakes of the infants were consistently above RDI (Table 5). Milk and infant cereals were the primary sources of the two nutrients. During the first year, the mean calcium:phosphorous ratio in

TABLE 5

Mineral Content in Diets of Non-Breastfed Infants

Age (months)	Calcium			Phosphorous			Iron			Sodium			Potassium		
	Mean	Sd (mg)	% RDI	Mean	Sd (mg)	% RDI	Mean	Sd (mg)	% RDI	Mean	Sd (mg)	% RDI	Mean	Sd (mg)	% RDI
1	475	186	95	354	142	141	4.3	5.1	61	167	75	132	444	278	176
3	622	265	124	485	227	194	9.5	8.8	136	220	130	122	691	412	192
5	818	337	164	671	286	268	11.8	8.8	169	370	203	171	1127	570	261
6	912	322	182	771	275	308	12.3	9.3	176	456	220	198	1371	584	329
7	987	319	197	862	270	215	12.2	8.8	174	548	243	---*	1582	566	---*
8	1007	322	202	900	272	225	12.0	8.3	171	614	255	242	1669	509	328
10	964	310	193	908	257	226	10.5	7.6	150	854	353	312	1780	484	325
12	928	301	186	916	253	183	9.2	7.1	115	919	396	317	1828	485	315
15	853	290	171	900	241	180	8.1	6.5	101	1047	387	335	1865	524	299
18	811	303	162	895	264	179	7.1	4.5	89	1150	402	345	1887	534	283

* Body Weight of the Infants at 7 months was not determined

the diet was greater than 1 to 1. After the first year more phosphorous than calcium was present in the diets. This was mainly due to the greater ingestion of meat, which was high in natural phosphorous content, and the lower intake of both milk and infant cereals.

Except at 1 and 18 months, the mean iron intake was above RDI (Table 6). The main source of dietary iron was iron-fortified infant cereals. Milk was the secondary source up to 7 months, beyond that meat was the secondary source.

TABLE 6
Vitamin content in Diets of Non-breastfed Infants

Age (months)	Vitamin A			Thiamin			Riboflavin			Niacin			Vitamin C		
	Mean	Sd. (iu)	% RDI	Mean	Sd (mg)	% RDI	Mean	Sd (mg)	% RDI	Mean	Sd (mg)	% RDI	Mean	Sd (mg)	% RDI
1	2428	1845	182	0.46	0.25	156	0.73	0.43	184	4.9	2.3	99	44.5	17.6	222
3	4267	2765	320	0.66	0.32	220	1.05	0.55	263	6.7	3.3	135	62.6	24.6	313
5	5880	3198	441	0.74	0.39	249	1.42	0.67	357	7.6	4.0	154	71.1	26.8	355
6	6542	3737	491	0.76	0.36	253	1.60	0.63	402	8.1	4.5	164	72.7	28.2	363
7	6730	3314	505	0.77	0.34	155	1.76	0.62	294	8.8	4.7	148	77.3	32.9	386
8	6775	4076	508	0.79	0.39	159	1.81	0.65	303	9.2	4.7	154	80.7	40.7	403
10	6714	3560	504	0.75	0.34	151	1.76	0.60	295	10.1	5.1	169	91.6	46.1	458
12	6210	3543	466	0.73	0.44	105	1.70	0.58	213	11.0	5.5	123	107.2	89.2	536
15	5737	3476	430	0.74	0.44	107	1.69	0.90	212	12.3	5.4	137	113.1	70.7	565
18	5794	3702	434	0.80	0.51	115	1.66	0.69	208	13.6	6.4	151	120.2	81.8	601

The mean dietary levels of sodium and particularly potassium were well above RDI (Table 5). From 10 months onwards, sodium intake was over 300% RDI. Potassium intake was consistently high. By the sixth month the mean intake exceeded 300% RDI. During early infancy milk was the primary source of sodium. By around 8 months table foods provided most of the dietary sodium. Milk was the primary source of potassium during the entire 18 months. Fruit and fruit juices were the secondary and vegetables the tertiary sources of potassium.

The mean vitamin intakes, particularly Vitamins A and C, were above RDI (Table 6). The primary source of Vitamin A was vegetables. This was followed by vitamin supplements, which provided, on the average 30% of the total daily dietary Vitamin A. In the first 3 months, milk provided more than 50% of the dietary thiamin. When infant cereals were established in diet, they assumed the primary source of thiamin. In the latter months, meat and vitamin supplements contributed 10 to 20% of the dietary thiamin respectively. The mean riboflavin content in the diets was in excess of RDI. Throughout the 18 months milk was the primary, infant cereals the secondary and meat the tertiary sources of riboflavin. Similarly niacin was above recommendation. During the first 6 months, milk and cereal contributed most of the vitamin. As meat intake increased so did niacin. Vitamin C intake increased continually throughout the 18 months. By 18 months, the mean Vitamin C content in the diets was 120 mg. or 600% RDI. Fruit and fruit juices provided most of this vitamin. Vegetables contributed about 10% and vitamin supplements, between 20 to 30%.

DISCUSSION

The types and quantities of foods consumed by infants varied widely. This was reflected in the wide variation in energy and nutrient intakes. Nevertheless, after the initial rapid rise, the amount of food consumed stabilized by about 10 months of age. In contrast, energy consumption continued to rise. This could be explained by the energy density of the diets. During the first 8 months when food consumption rose noticeably, there was little change in energy density of the diets. Subsequently, when adult foods, which were generally more energy dense than infant foods, were introduced and quickly assumed prominence in the infant's diets (7), the energy density of the diets increased. As a result, energy consumption increased without a parallel rise in the volume of food consumed. This observation supports Fomon's (8) contention that infants eat according to energy need and not by volume of food.

The current RDI for energy is high. Except in the first month, the mean energy intake was much lower than the RDI. However, the mean body weight and heights of the infants were close to the 50th percentile of the National Center for Human Statistics values for weight and height. Thus the infants were eating sufficient foods for normal growth and suggested that the current RDI is high and should be lowered. The RDI for energy was estimated from an asuumed intake of 850 ml./day of breastmilk of 75 kcal/100 ml. by infants who were growing normally (10). More recent studies showed that the normal breastmilk volume averaged between 600 to 750 ml./day (11) and the caloric density was 65 kcal/day (12). Obviously both these figures are much lower than the figures used to estimate energy requirements of infants. This explains the overestimation of the RDI for energy.

Energy contributed by dietary fat between 6 and 12 months was below recommended level of 30%. This could be explained by the large number of infants being fed low-fat, 2% and skimmed, cow's milk. From 6 months onwards approximately 70% of the infants were fed 2% and skimmed milks (3). Infants fed the defatted milks consumed the same amount of energy per day, but significantly more food and protein and less fat than infants who were fed formula or whole cow's milk (13). If all the infants were fed whole cow's milk or formula, the mean percent of energy derived from fat would be greater than 30% at all times. After 12 months, fat contributed more than 30% of the dietary energy mainly because of the presence of oil and fat and fat containing table foods. The clinical significance of the low fat intake from 6 to 12 months remains to be

determined.

A low fat diet has low satiety value and therefore encourages consumption of greater amounts of food. Although there is no clinical evidence that indicates a low fat and high protein diet is harmful, it potentially contains too little essential fatty acids and excessive renal solute load. Therefore, for optimal nutrition 2% and skimmed milk are not recommended during the first year of life (14).

The relatively low mean intake of iron after the first year of life is of concern. In an earlier paper, the incidence of inadequate iron intake was reported (15). Although the mean nutrient intakes were close to or above RDI the distribution of intake was skewed to lower levels of intake. As a result, 53 to 70% of the infants received less than the RDI for iron during the last six months of the survey. Biochemical data are therefore needed to determine the prevalence of iron deficiency in Canadian infants.

Inadequate intake of iron-rich foods such as iron-fortified infant cereals was the main reason for the low iron intake. Although, almost all infants were eating cereal products, many were eating adult-type cereals that were fortified with iron at a much lower level than infant cereals. The quantity of consumption of these products were not sufficiently high to meet the RDI for iron. The recommendation of the American Academy of Pediatrics (16) to include infant cereals in the diets of children up to 2 years of life is well advised.

Among infants older than 10 months, the mean sodium intake was 300% or more above RDI. Excessive intake of sodium is not recommended since it contributes to a high renal solute load which may have an adverse effect on individuals who are predisposed to hypertension (17). The main source of sodium in diets of infants whose dietary intake of sodium was high was home-made foods. Much of the sodium was dervied from table salt or commercial canned foods (18). Salt-free commercial baby foods contributed no more than 20% of the dietary sodium. Kerr Jr. et al (19) and Nichols (20) have reported similar observations. Hence, to minimize the risk of excessive sodium intake, salted table foods should be avoided, at least during the first year of life.

Vitamin supplements were largely unnecessary. Many infants were consuming levels of Vitamin A that might be considered excessive. Prolonged intake of excessive Vitamin A can be hazardous (21). The main sources of the vitamin in the infants' diets were vegetables, such as carrots and squash, and vitamin supplements. Food alone provided more vitamin A, mostly in the form of carotene, than recommended. Vitamin supplements provided an additional 30% of the dietary vitamin A. The same was true for Vitamin C. Fruit and fruit juices, such as orange juice and vitamin-C fortified fruit juices, contributed more than enough vitamin C to satisfy RDI. Although Vitamin C is water soluble and is not known to be toxic at the observed levels of intake, excessive intake of this vitamin is unnecessary. Since in both cases foods alone provided more than enough of the vitamins to meet RDI, vitamin supplements containing these 2 nutrients are largely unnecessary.

The observed nutrient contribution of the various food groups emphasized the importance of milk as the foundation of nutrition, especially during early infancy. As the infants grew, other foods were added to the diets to complement milk. Infant cereals, which are normally the first solid foods to be introduced were important to the iron nutriture of infants. In fact, without iron-fortified infant cereals, the RDI for iron was found to be difficult to meet (16). Besides iron, infant cereals provided valuable amounts of calcium, phosphorous, thiamin, riboflavin, and niacin as they were fortified with these nutrients. Fruit and fruit juices were excellent sources of Vitamin C and vegetables were excellent sources of Vitamin A. In later infancy, as milk intake declined, and meat intake rose, the latter assumed greater importance as a source of protein, fat, niacin, phosphorous and other nutrients. In summary, the results stressed the importance of providing infants with diets comprising the four basic food groups. Variety within each group should be selected to provide a balanced intake of nutrients, that is, to avoid excessive or inade-

quate intake of specific nutrients.

CONCLUSION

The dietary intakes of the infants in the present survey provided mean levels of nutrients that were close to or above the RDI. In spite of the low energy intake, relative to the RDI, the infants grew at normal rates. This has suggested that the RDI for energy is high and should be lowered. Certain problems in the diets were identified, most of which were related to older infants. These included: (1) lack of adequate iron due to early termination of infant cereal feeding, (2) high intake of sodium related to introduction of salted-table foods and (3) excessive intake of Vitamins A and C, much of which were from vitamin supplements. The following recommendations are suggested for correction of the problems. (1) Feed iron-fortified infant cereals up to 2 years of age, (2) avoid feeding salted-tabled foods or alternatively continue to use salt-free commercial baby food up to the first year of life, (3) Dispense with vitamin supplements that contain vitamins A and C, and (4) feed variety of foods in the 4 basic food groups.

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DIETARY ENERGY AND NUTRIENT INTAKES AND GROWTH OF HEALTHY AUSTRALIAN INFANTS IN THE FIRST YEAR OF LIFE

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ABSTRACT

A prospective study of growth and nutrition in Australian infants has shown a high rate of breast-feeding, which was continued past 6 months in almost two-thirds. This trend back to breast-feeding was most pronounced in the highest social class. Protein intakes were much less in the first 6 months than in earlier studies of predominantly artificially fed infants. Intakes of sodium were less than recent reported figures. Body weight increases in this group were considerably below those reported from infants in Australia when artificial feeding prevailed.

KEY WORDS: Infant nutrition, breast-feeding, growth

INTRODUCTION

Nutritional studies are usually cross-sectional in nature. The scarcity of longitudinal studies of growth and feeding practices of healthy infants and young children is partly responsible for continuing controversies about what is "normal" (1). This also complicates the task of comparing results from studies undertaken with different racial groups in different environments (2). In an attempt to close these gaps in knowledge we have commenced a prospective, 5-year-long study of healthy infants born in Perth. The present paper incorporates the main findings about nutrient and energy intakes and somatic growth of these infants in their first 12 months.

MATERIALS AND METHODS

In common with North America and Western Europe, Australia is a highly urbanized, technologically advanced society and shares with them the so-called

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Western styles of living, health and disease. This study was done in Perth, a city of 850,000 situated on the coast of Western Australia of which it is the State capital.

The study began in 1979. One-hundred-and-four boys and 101 girls born in Perth were selected randomly from the "Midwife Record of Birth" data of the Western Australian Community and Child Health Services.

All subjects were -

(a) at least second generation Australian born from two-parent families, but excluding Australian Aborigines,

(b) full-term singletons from normal pregnancies and deliveries, weighing 2500 g or more at birth.

Subjects randomly selected, but not meeting these criteria, were excluded from the study.

The subjects were mostly from middle class families; 75% of them were from the middle two groups of a 4-point social scale (3).

Birth weight and length were available for all infants from midwife records. Subsequent measurements were made at 6 weeks, and at 3, 6, 9 and 12 months using standard methods. At 6 weeks of age, information about the type and frequency of milk feeding and the type, amount and frequency of feeding non-milk foods was obtained from the mother by recall. At later times, an accurate record of amounts of all food and fluids other than breast-milk consumed by the infant was kept by the mother for the 7 consecutive days before the physical measurements were done. This allowed cross-checking of the record then. If the mother was breast-feeding, she noted this fact.

Calculation of energy and nutrients from foods other than breast-milk was made using a computerized programme based on Australian food tables (4). The mean total energy intakes of infants at ages up to 6 months have been *estimated* from energy requirements calculated for maintenance and growth (5). These *estimates*, expressed per kilogram body weight, are in good agreement with requirements calculated by Clements (6). They are similar to intakes reported by Beal (7) and Fomon et al. (8) and are a *guide* to total energy intake up to this age.

RESULTS

Feeding Practices: At 6 weeks of age, 83% of infants were being breast-fed, on demand, including 4% being complemented. By 6 months, this percentage had fallen to 64% including 8% with a formula complement, and at one year of age, 25% of the group continued to be breast-fed to some extent. Breast-feeding was more common in the upper two social groups throughout the 12 months (Table 1).

Low-solute ("humanized") formulae* were mostly used for those not breast-fed. After 6 months, use of low solute formulae decreased and cow's milk increased.

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* S26, SMA : Wyeth Pharmaceuticals Pty. Ltd., Sydney, Australia.
Lactogen, Nan : The Nestlé Company (Aust.) Ltd., Sydney, Australia.
Enfamil : Mead Johnson (Bristol Myers), Sydney, Australia.

TABLE 1

Percentage of Infants Breast-Fed

Age	Percentage of total Sample	Percentage of each social group*			
		A	B	C	D
6 weeks	83	96	97	83	52
3 months (13 weeks)	77	96	90	69	52
6 months (26 weeks)	64	88	80	53	46
9 months (39 weeks)	46	68	58	35	29
1 year (52 weeks)	25	27	39	17	13

* highest A → lowest D

At 6 weeks of age 10% of infants were given foods other than breast milk or formula. This was usually water, fruit juice or a sweetened vitamin C concentrate. At 3 months, the percentage has risen to 37%; the foods given were usually small and irregular amounts of fruit juices, cereals and puddings. By 6 months, 96% were being given foods such as fruits and fruit juices, vegetables, cereals, milk puddings and meat dinners. These foods were introduced later to breast-fed babies (9).

Weight and Lengths: The mean body weights and lengths of boys and girls at birth, 6 weeks, and 3, 6, 9 and 12 months are shown in Table 2.

Energy and Nutrient Intakes: *Estimated* energy intakes progressively increased from 550 and 510 kilocalories (2300 and 2135 kilojoules) for boys and girls respectively at 6 weeks, to 615 and 580 kilo-calories (2570 and 2430 kilojoules) at 3 months, and 740 and 700 kilocalories (3100 and 2930 kilojoules at 6 months.

Based on a value of 70 kilocalories (294 kilojoules) per 100 ml. breast milk (4) or low solute formula and calculations from recorded amounts of solid foods eaten, protein intakes over this period rose from approximately 9.9 and 9.2 g per head daily for boys and girls at 6 weeks, to 11.6 and 10.8 g at 3 months, and 18.4 and 15.1 g at 6 months.

Food energy, and intakes of protein, fat, carbohydrate, and of some minerals and vitamins, are shown in Table 3 for infants who were never breast-fed or no longer were at 9 and 12 months.

Sodium Intakes and Salt Usage: Daily sodium intakes calculated from estimates of breast-milk or formula, and from recorded food intakes rose from 5mEq at 6 weeks to 6mEq at 3 months and 13mEq at 6 months of age. At 9 and 12 months, mean daily sodium intake calculated from food records was 38mEq and 53mEq.

These sodium figures do not include any from salt added to food during its preparation at home. At 3 months less than 2% of mothers added salt to their infant's food. This had increased to 27% at 6 months, 42% at 9 months and 56% at 12 months.

TABLE 2

Weights* and Lengths∇ of Infants, Birth to 12 Months

Age	Boys			Girls		
	n	Weight	Length	n	Weight	Length
Birth	104	3.56 ± 0.04	51.6 ± 0.2	101	3.47 ± 0.04	50.7 ± 0.2
6 weeks	95	4.81 ± 0.06	56.3 ± 0.2	96	4.60 ± 0.05	55.5 ± 0.2
3 months	99	5.92 ± 0.08	60.7 ± 0.2	96	5.61 ± 0.06	59.3 ± 0.2
6 months	95	7.85 ± 0.09	67.5 ± 0.3	94	7.44 ± 0.08	65.9 ± 0.2
9 months	88	9.11 ± 0.10	72.1 ± 0.3	90	8.56 ± 0.09	70.6 ± 0.2
12 months	85	10.01 ± 0.11	75.9 ± 0.3	88	9.50 ± 0.10	74.9 ± 0.2

* Mean values (kg) ± S.E.M.

∇ Mean values (cm) ± S.E.M.

DISCUSSION

The present study has shown a high incidence of breast-feeding which continued into the second half of infancy for more than half the subjects. This appears to confirm a recent return towards breast-feeding (10-13) and presents an excellent opportunity to examine the nutritional consequences of this trend.

The increase in breast-feeding, and the widespread use of formulae designed to simulate it ("humanized"), has resulted in protein intakes which are significantly lower than when cow's milk-based formulae were the prevalent feedings. For example, the mean daily protein intakes of infants aged 6 months in an earlier Australian study (14) was 36 g compared to the estimated 18.4 g for boys and 15.1 g for girls in this study. By 12 months of age, when mixed feeding had become well established, these differences were much less; 44 g per day in the earlier study and 38.5 g for boys and 38.1 g for girls in the present one. The U.S. Department of Agriculture (15) has noted a 40% decline in protein intakes in 1977-78 in comparison with 1965 and attributed this to changes in composition of infant formulae. It was also noted that iron intakes for infants in the U.S. have doubled since 1965, apparently because of iron fortification of baby cereals and infant formulae.

TABLE 3

Energy and Nutrient Intakes of Infants aged 9 Months and 12 Months
(Mean per day ± S.E.M.)

	9 Months		12 Months	
	Boys n = 46	Girls n = 52	Boys n = 62	Girls n = 63
Energy*	895 ± 23 (3745 ± 95)	865 ± 24 (3620 ± 100)	990 ± 26 (4145 ± 108)	950 ± 25 (3975 ± 105)
Protein (g)	33.8 ± 1.2	32.8 ± 1.4	38.5 ± 1.4	38.1 ± 1.3
Fat (g)	42.7 ± 1.8	39.6 ± 1.5	42.5 ± 1.6	42.4 ± 1.5
Carbohydrate (g)	103.4 ± 3.3	95.0 ± 2.8	115.9 ± 3.1	103.5 ± 3.4
Calcium (mg)	790 ± 39	838 ± 34	781 ± 38	797 ± 34
Iron (mg)	7.0 ± 0.7	7.5 ± 0.7	5.7 ± 0.3	5.7 ± 0.4
Thiamine (µg)	786 ± 49	691 ± 31	822 ± 41	760 ± 34
Riboflavin (mg)	1.6 ± 0.07	1.7 ± 0.07	1.6 ± 0.07	1.6 ± 0.05
Niacin∇ (mg)	6.4 ± 0.6	6.2 ± 0.5	7.0 ± 0.6	5.9 ± 0.3
Vitamin C (mg)	62.9 ± 6.3	55.3 ± 4.7	65.8 ± 5.0	45.8 ± 3.8

* kilocalories (kilojoules)

∇ this does not include niacin obtained from dietary tryptophane

The return to breast-feeding, with later introduction of solids, should reverse this trend.

Sodium intakes in this study were lower than those reported from the U.S. as recently as 1972 (16). This difference is due to the lower sodium content of breast-milk and "humanized" formulae compared to cow's milk and the greater use of home-cooked foods rather than canned baby foods. An increasing public awareness of the possible long-term implications of excessive salt intake for the development of degenerative cardio-vascular disease may also have contributed to these lower salt intakes although there is still some argument about the causality of this relationship for humans (16).

There were some interesting findings in the anthropometric data from the present study. Although birth weights for these infants were not significantly different from some earlier Australian figures (17, 18) growth data over the 12 months resemble those reported in Australia almost 50 years ago when breast-feeding prevailed (17) and are similar to those of a recent study from the Nursing Mothers' Association of Australia, a voluntary organization which promotes breast-feeding and offers help to mothers wanting to breast-feed (19). Weight gain over the year was significantly less for these Perth boys and girls than it was in another Australian study of the mid 1960s, particularly over the first 3 months (20), when milk-based formulae and the early introduction of solids prevailed, and when infantile obesity was increasing (21).

In a review of infant feeding, Wickes concluded that its history could be seen as a chronicle of man-made errors, some of which have resulted from the decline in breast-feeding (22). This was actually seen by Rueda-Williamson and Rose (23) as an advantage in studying infants in the first year of life, namely that "some of the difficulties encountered in evaluating nutrient intake are minimal during most of this period since intakes are essentially in ounces of milk and jars of food of established nutrient values". The present study strongly suggests that with the widespread return to breast-feeding and the more appropriate use of better formulated infant feedings by mothers who cannot or choose not to breast-feed, many of these errors will be self-correctiong.

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Inducer and Suppressor T Cell Subsets in Protein-Energy Malnutrition: Analysis by Monoclonal Antibodies

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ABSTRACT

Malnutrition results in impaired cell-mediated immune responses. We have examined various subsets of T lymphocytes reactive with monoclonal antibodies against cell-surface antigens using flourescein-triggered cell sorting technique. There was a reduction in the absolute number of T lymphocytes reactive with anti-T3. Both inducer cells identified by anti-T4 and suppressor cells identified by anti-T8 were decreased in number, particularly the former. The inducer/suppressor ratio was low. These alterations in lymphocyte subset numbers were reversed to normal following nutritional therapy for 4-8 weeks. Changes in T lymphocyte subpopulations may partly explain depressed cell-medicated immunity in malnutrition.

KEY WORDS: Lymphocyte subsets, Inducer cells, Suppressor/cytotoxic cells, Cell-mediated immunity, Protein-calorie malnutrition.

INTRODUCTION

Protein-energy malnutrition is generally associated with an increased frequency and severity of infection (1). Both children and adults with the syndrome of kwashiorkor and marasmus show atrophy of lymphoid organs, particularly the thymus, and the pattern of microorganisms isolated from malnourished subjects resembles the spectrum of pathogens found in patients with primary immunodeficiency disorders. These clinical and autopsy observations led to systematic studies of the immunocompetence of nutritionally deprived individuals. Many types of immune responses are variably impaired in deficiencies of proteins, energy, vitamins and trace elements (2-5). Consistent findings include depressed cell-mediated immunity documented by impaired delayed cutaneous hypersensitivity reactions (6,7), decreased number of rosette-forming lymphocytes (8-10), lowered in vitro lymphocyte stimulation response to mitogens especially in the presence of autologous plasma (11,12), and reduced thymic hormone activity (13,14). The availability of monoclonal antibodies reactive against surface antigens of lymphocytes has revealed antigenically and functional distinct subsets of T cells (15,16). We report alterations in T lymphocyte subsets analyzed by monoclonal antibodies in a group of young children with protein-energy malnutrition.

PATIENTS AND METHODS

Six infants and children, aged 10 to 32 months, were diagnosed to have primary protein-energy malnutrition on the basis of reduced dietary intake,

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weight-for-age and weight-for-height below 70 percent of the 50th centile of standard for chronological age, and clinical manifestations. The presence of active concurrent or recent infection was excluded by clinical examination, chest x-ray, negative Limulus assay for endotoxemia and normal levels of C-reactive protein. Blood was drawn on first examination and 4-8 weeks after nutritional therapy. The latter provided approximately 150 Kcal per kg body weight and 3.5 g protein per kg body weight.

Peripheral blood mononuclear cells were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation. Three monoclonal antibodies with specificities for T3, T4 and T8 cell subpopulations were used (Orthoclone ®, Ortho Pharmaceutical Corporation, Raritan, NJ). The antisera were reactive by complement-mediated cytolysis and/or indirect immunoflourescence to a titer from 1: 100,000 to 1: 500,000. Cytoflourographic analysis of lymphocytes was performed by indirect immunoflourescence with flourescein-conjugated anti-mouse IgG (Meloy Laboratories, Springfield, VA) on System 50H Cytoflourograph Cell Sorter. Statistical analyses included Student's t test and paired t test.

RESULTS AND DISCUSSION

The patients showed clinical features characteristic of protein-energy malnutrition, namely growth failure, apathy and/or irritability, brittle hypopigmented easily pluckable hair, skin changes and in 2 patients, pitting edema. Biochemical analyses (Table 1) supported the diagnosis of malnutrition. Deficits of vitamins and trace elements were excluded by normal cell indices and morphology, blood levels of folate, ferritin, vitamin B12 and zinc within the normal range, and normal transferrin saturation. Clinical and biochemical recovery (Table 1) was evident at the time of second examination.

Table 2 describes the surface characteristics of lymphocytes from malnourished patients before and after nutritional therapy and compares them with those from healthy controls of the same age group. There was an absolute reduction of the total T lymphocytes reactive with anti-T3. Both inducer (identified by reaction with anti-T4) and suppressor (showing reactivity with anti-T8) subsets decreased, particularly the former. The inducer/suppressor ratio was lower before nutritional therapy was started and rose thereafter. The small size of the study group did not permit analysis of T lymphocyte subsets in relation to severity of malnutrition. Individual nutrient deficiencies had been excluded by clinical and biochemical examination. It is well recognized that deficits of vitamins and trace elements suppress immune responses through a variety of pathogenetic mechanisms, including decreased DNA and RNA synthesis, impaired maturation and differentiation as a result of decreased thymic factor activity, altered purine salvage pathway, disturbed hormonal homeostasis, changed homing patterns, etc. Our unpublished data on patients with acrodermatitis enteropathica with zinc malabsorption indicate perturbations in T cell subsets correctable by zinc supplements. Laboratory animals deprived of pyridoxine, vitamin A, zinc, iron, copper and selenium exhibit changes in T cell number and function and host response to challenge with *Salmonella typhimurium*, *Listeria monocytogenes* and Coxsackie B3.

Optimal immune response depends upon complex cellular interactions, both helper and inhibitory (17-19). Abnormalities of immunoregulatory T cells have been reported in a variety of human diseases, for example immunodeficiency states, autoimmune diseases, graft-versus-host reaction, and cancer (20-23).

Previous studies have documented a general reduction in T cells in protein-energy malnutrition (8,9). In order to determine whether imbalance among subsets of human T lymphocytes exists in malnourished children, we

TABLE 1

Nutritional and biochemical indices and lymphocyte number

Group	Weight-for-age* (%)	Weight-for-height* (%)	Absolute lymphocyte count per mm^3	Serum Concentration			
				Albumin (g%)	Prealbumin (mg%)	Transferrin (mg%)	Retinol-binding protein (mg%)
Protein-energy malnutrition							
I BT (n=6)	67±4.1	59±3.7	2,136±439	2.60±0.50	9±2.1	112±19	1.9±0.6
II AT (n=5)	79±3.9	82±5.6	2,410±561	3.47±0.33	27±5.6	201±27	5.8±1.3
Well nourished controls							
III (n=10)	93±5.7	98±4.2	2,605±380	4.11±0.29	29±4.7	191±31	6.1±1.1
Significance p							
I vs II	<0.05	<0.01	NS	<0.05	<0.005	<0.05	<0.001
I vs III	<0.01	<0.01	NS	<0.01	<0.005	<0.05	<0.001
II vs III	<0.05	<0.05	NS	<0.05	NS	NS	NS

Values are given as mean ± SEM BT Before therapy AT After therapy NS Not significant

*Expressed percentage of the 50th centile of standard for age (National Center for Health Statistics Data)

TABLE 2

T lymphocyte subsets in malnourished children before and after dietary therapy and in well nourished controls

	Reactivity with monoclonal antibodies							
	T3		T4		T8		T4:T8 ratio	
	Before	After	Before	After	Before	After	Before	After
Malnourished			%					
1	41	66	13	41	17	25	0.76	1.64
2	39	69	17	43	19	23	0.89	1.87
3	42	74	20	44	18	22	1.11	2.00
4	31	61	12	37	16	21	0.75	1.76
5	40	ND	17	ND	14	ND	1.21	ND
6	26±2.5	65.0±2.7	15.0±6.5	40.8±1.2	17.6±0.05	22.0±0.8	0.87±0.11	1.86±0.07
	p<0.001		p<0.001		p<0.005		p<0.001	
Well nourished								
(n=10)	67.0±4.3*		41.0±3.7		25.0±2.3		1.98±0.21	

* Values are given as mean ± SEM
ND Not done

analyzed peripheral blood mononuclear cells of these patients using monoclonal antibodies to the stable surface glycoprotein antigens of inducer and suppressor T cell subsets. A marked reduction in the number of T4 inducer cells was observed whereas the number of T8 suppressor cells was decreased to a lesser extent, thereby altering the T4:T8 ratio. The changes were reversed by nutritional supplements given for a period of 4-8 weeks. These data bear relevance to the observations of increased prevalence and severity of infections, cutaneous anergy, and reduced antibody response to T-responsive antigens. Our recent work, in preparation, shows a reduction in the number as well as function of T helper lymphocytes. There is inconclusive evidence on the incidence of cancer and autoimmune disease among malnourished groups relative to well nourished populations. Clearly more work is needed in this important area.

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Brief Communication

IMMUNOGLOBULIN AND PROTEIN LEVELS IN BREAST MILK PRODUCED BY MOTHERS OF PRETERM INFANTS

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ABSTRACT

Concentrations of total protein and immunoglobulins A, G and M were determined in milk samples collected from 21 mothers who delivered preterm and 21 mothers who delivered at term. Levels of protein and of immunoglobulins, particularly IgA, were significantly higher in the preterm group samples. There were no differences in concentrations of protein or individual immunoglobulins related to mode of expression of manual, manual or mechanical, rate of milk flow or time of collection during the 24-hour period. It is suggested that milk produced by mothers of preterm infants is unique in its nutritional and immunological contents for the increased needs of low birth weight infants. Thus, mother's milk is the ideal source of nutrition and protection for all infants irrespective of their birth weight or gestation.

KEY WORDS: Breast feeding, human milk, immunoglobulins, protein, prematurity, low birth weight infants

INTRODUCTION

Renewed interest in breast feeding has stimulated many investigations of the nutritional and immunologic contents of human milk (1-4). While breast milk is accepted as the preferred exclusive source of feeding the healthy term infant, there has been some concern expressed about the nutritional adequacy of human milk for the growth needs of the preterm low birth weight neonate. Even though the infant may be too weak to suckle, it is possible to express milk by hand or by a mechanical pump and give it to the baby by gavage. Recent preliminary data indicate that early milk produced by mothers delivered of preterm infants is 20-30 % higher in total energy and lipid concentration and 15-20 % higher in total protein concentration compared to milk produced by mothers delivering at term (5,6). Our study confirms this.

Among the various protective factors present in breast milk, immunoglobulins particularly IgA, are the most important and well studied components. There are no data on the concentrations of immunoglobulins produced in milk from mothers giving birth prematurely. We have studied this and found significantly higher levels of IgA, IgG and IgM in preterm milk compared with term milk.

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OBSERVATIONS

We examined milk produced by 21 mothers who give birth between 28 and 35 weeks gestation (Mean 30.8 ± 2.7) and from 21 mothers who had delivered between 37 and 41 weeks gestation (Mean 39.4 ± 1.7). Morning milk samples were obtained by complete manual expression of both breasts on days 3, 7, 14, 28 and 56 postpartum. The participants were asked to wash their breasts with sterile deionized water before expressing the milk into sterile containers. In 5-7 mothers in each group, 24-hour milk collections were conducted as six 4-hourly emptying of breasts. Samples were stored at -40 C for upto four weeks before analysis.

Immunoglobulin levels were assayed on delipidated samples by double antibody radioimmunoassay using heavy chain monospecific reagents. Total protein was calculated by multiplying Kjeldahl nitrogen by 6.25; it has been suggested that this is a more appropriate factor than 6.38 since approximately 5 % of the total nitrogen of human milk is nonamino acid nitrogen (5). Analysis of covariance and *t* tests were done on log-transformed data.

Concentrations of protein and IgA were significantly higher in milk produced by mothers giving birth prematurely compared with samples from mothers

TABLE 1

Protein Levels

Day postpartum	Number	Term	Preterm	Significance
3	21	2.1 ± 0.17	2.4 ± 0.14	< 0.05
7	19	1.8 ± 0.11	2.3 ± 0.15	< 0.01
14	20	1.4 ± 0.11	1.9 ± 0.16	< 0.05
28	18	1.3 ± 0.10	1.5 ± 0.21	NS
56	18	1.0 ± 0.12	1.1 ± 0.16	NS

Data are shown as means ± standard error

NS Not significant

delivered at term, particularly during the early phases of lactation (Table 1). Levels of IgA were significantly higher throughout the lactation period studied in preterm versus term samples (Table 2). Concentrations of IgG and IgM did not differ in the milk of the two groups of mothers on days 3,7 and 56, but were higher in the preterm group on days 14 and 28 (Table 2).

There were no differences in concentrations of protein or individual immunoglobulins related to mode of expression of milk, manual or mechanical, rate of milk flow or time of collection during the 24-hour period (data not shown).

TABLE 2

Immunoglobulin Levels

Day postpartum	Number	Immunoglobulin A			Immunoglobulin G			Immunoglobulin M		
		Term	Preterm	*p*	Term	Preterm	*p*	Term	Preterm	*p*
3	21	112 ± 23	261 ± 49	<0.01	2.5 ± 0.41	2.7 ± 0.57	NS	7.9 ± 2.1	8.2 ± 2.1	NS
7	19	71 ± 19	212 ± 33	<0.01	2.0 ± 0.37	2.4 ± 0.31	NS	6.3 ± 1.8	8.1 ± 1.9	NS
14	20	60 ± 17	125 ± 23	<0.01	1.9 ± 0.43	2.9 ± 0.39	<0.05	7.7 ± 2.3	11.9 ± 2.3	<0.05
28	18	39 ± 14	96 ± 25	<0.05	2.1 ± 0.39	3.2 ± 0.53	<0.05	3.7 ± 1.8	6.5 ± 2.7	<0.05
56	18	46 ± 17	91 ± 21	<0.05	1.9 ± 0.59	2.9 ± 0.47	NS	2.8 ± 1.1	3.9 ± 1.8	NS

Data are shown as geometric means ± standard error

NS Not significant

COMMENTS

Human milk provides effective passive immunity during the crucial first few days after birth when both systemic and local mucosal immunity is low. Breast-fed infants have significantly lower incidence of infections, both serious potentially life-threatening ones and relatively minor ones (3,7). Low birth weight infants are at greater risk of infectious complications, due in part to delayed maturation of host defenses including gut immunity.

Our observation of increased levels of protein and immunoglobulins, especially IgA, in milk produced by mothers of preterm infants indicates that mother's milk would be the ideal source both of nutrition and passive protection. IgG and IgM levels also were higher in milk from preterm group on days 14 and 28; however, the functional significance of these two immunoglobulins on mucosal surfaces is not clear except when IgA is absent. On the other hand, IgA antibodies in ingested breast milk have been documented to prevent mucosal attachment of a variety of intestinal pathogens and alter the in vitro colony characteristics and agglutinating ability of certain enteropathogenic bacteria.

Previous considerations of the adequacy of human milk for the low birth weight infant have utilized information derived from pooled human milk, mostly from mothers delivering at term, which led to the erroneous recommendations that breast milk may not be ideal for the small infant. Our studies and other recent observations cited above indicate that milk produced by the mother of the preterm infant is uniquely suited for the increased nutrient and immunologic requirements which may more than compensate the limitation imposed by the reduced volume he can tolerate. Thus, mother's milk is the ideal source of nutrition and protection for all infants, irrespective of their birth weight or gestation.

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PRODUCTION OF LABELLED 25 HYDROXYVITAMIN D FROM ^{14}C VITAMIN D AS A MARKER OF LIVER FUNCTION AFTER 65 PER CENT HEPATECTOMY IN DOGS .

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ABSTRACT

In order to appreciate hepatocyte activity after 65 per cent hepatectomy, plasma ^{14}C 25-hydroxyvitamin D (^{14}C 25-OH-D, % total dose x litre $^{-1}$ x body weight -kg-) was measured following an intraportal bolus injection of 4 µCi ^{14}C vitamin D.
As disturbance of the spleno-pancreatic venous flow might lead to post operative hepatic atrophy, the hepatectomized dogs were separated into three groups : no shunt (group I,n=8), mesocaval (group II, n=3) or portocaval shunt (group III, n=8). As compared with five controls, the ^{14}C 25-OH-D level at day 6 was within the normal range (mean ± SEM = 231 ± 21) in groups I (187 ± 28) and II (184 ± 12) and was significantly depressed in group III (114 ± 14, $p<0.01$).
The results were compared with post operative proaccelerin levels, which were decreased in group III (61 ± 9 %) and normal (100 %) in the other groups. From the present results, it appears that 25 hydroxylation is a reliable index of liver function and that portacaval shunts impair liver activity after 65 per cent hepatectomy. The latter finding could result either from a major reduction in hepatic blood flow or suppression of such hepatotrophic factors as pancreatic hormones.
KEY WORDS : vitamin D-25 hydroxy vitamin D - proaccelerin - 65 % hepatectomy - mesocaval shunt - portocaval shunt - liver function.

INTRODUCTION

Extensive liver resection might play a role in the future treatment of hepatic cancer provided that severe hepatic insufficiency could be prevented in the post operative period. Liver regeneration after subtotal hepatectomy is usually gauged in terms of CH_3-^{3} H thymidine incorporation (1,2), hepatocyte DNA content and mitotic index, while liver function is estimated from circulating levels of albumin and coagulation factors such as proaccelerin and proconvertin(3,4). Metabolic function may also be used as an index of hepatic activity. For instance, it is well known that 25 hydroxylation of vitamin D is a liver specific function. Accordingly, we studied the production of labelled 25-hydroxyvitamin D (25-OH-D) from ^{14}C vitamin D injected into dogs after subtotal hepatectomy. The results were compared with proaccelerin levels in order to assess the validity of this index of liver function. Furthermore, as both trophicity and regeneration seem

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to depend on factors such as blood flow (5,6) and availability of trophic hormonal or non hormonal substances arising from the pancreas or the intestine (7-14), we studied the conversion of ^{14}C vitamin D to its 25 hydroxylated derivative in three different experimental conditions : after a 65 % hepatectomy alone or combined with either mesocaval or portacaval shunt.

MATERIAL AND METHODS

Experimental protocol

Twenty four mongrel dogs (mean body weight ± SEM= 22 ± 1 kg; mean plasma 25-OH-D levels ± SEM = 70 ± 7 nmole/litre) were divided into four groups and submitted under general anesthesia to the following surgical procedures: in group I (n=8): the hepatic mass was reduced to 35 % by resecting liver segments II, III, IV and V which correspond respectively to left lateral, left central, caudate and right central lobes (15). In group II (n=3) : the 65 % hepatectomy was combined to an end to side mesocaval shunt. The splanchnic blood stream was diverted from the liver while gastrosplenic and duodeno-pancreatic blood supplies to the liver remained unaffected. In group III (n=8): the 65 % hepatectomy was associated with a total portacaval shunt. The latter was obtained by a 2 cm large side to side portacaval anastomosis and ligation of the portal vein in the hilum of the liver (ECK's fistula). Group IV consisted of 5 sham-operated dogs. At the end of the operative procedure, 4 µCi of ^{14}C vitamin D were injected as a bolus into the portal vein. Plasma ^{14}C vitamin D, ^{14}C 25-OH-D and proaccelerin concentrations were determined daily for seven days or more.

Analytical procedures

Both (4-^{14}C) vitamin D_3 (specific activity 54 mCi/nmole) and (26-27-^{3}H) 25 hydroxyvitamin D_3 (specific activity 22 Ci/mmole) were purchased from the Amersham Radio Chemical Centre (U.K.). Unlabelled 25 hydroxyvitamin D (25-OH-D) was kindly supplied by ROUSSEL UCLAF (France).

Plasma 25-OH-D levels were measured by a competitive protein binding assay using normal diluted (1:5000) rat serum as binding protein (16). Before assay, samples of plasma were extracted (0.5 ml) with chloroforme-methanol (2:1). The 25-OH-D was separated from vitamin D by chromatography on silica gel (Serva 50-100 mesh) columns (0.5x6 cm). Elution was obtained by using 7 ml of either diethyl ether-light petroleum (1:2 V/V) or diethyl ether.

Plasma ^{14}C vitamin D and ^{14}C 25-OH-D levels (expressed as percentage of the total dose per litre of plasma and corrected by the body weight) were respectively measured by counting the total radioactivities of the less and more polar fractions, successively eluted by the diethyl ether-light petroleum and the diethyl ether.

Statistical analysis

The data were compared using the WILCOXON ranking test which does not require normal distribution and large sample sizes.

Results

The concentration-time curves for plasma ^{14}C vitamin D were similar in the four groups of dogs (fig. 1).

The concentration-time curves for plasma ^{14}C 25-OH-D were similar in groups I and II and not significantly different from controls (fig. 2). On the contrary, plasma ^{14}C 25-OH-D concentrations were significantly diminished in group III as compared with controls. Statistical differences were particularly significant at days 6 and 7 after hepatectomy ($P < 0.01$).

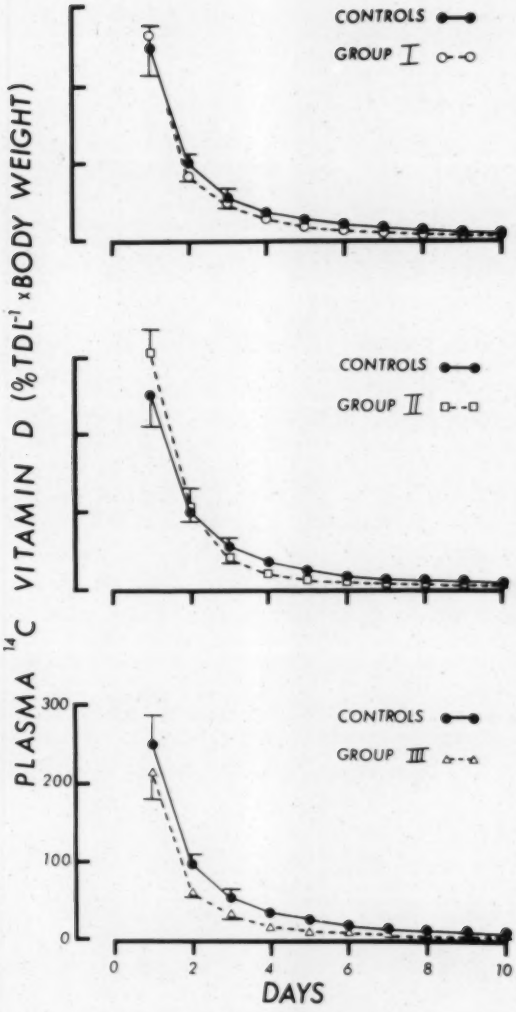

FIG. 1

Concentration-time curves for systemic plasma ^{14}C vitamin D after intra portal injection of ^{14}C vitamin D (per 100 of total dose per litre corrected by body weight (kg). Comparison in 4 groups of dogs : Controls (●—●, n=5), 65 per cent hepatectomized animals (i) without shunt : group I (○- -○, n=8), (ii) with mesocaval shunt : group II (□--□, n=3), (iii) with portacaval shunt : group III (△--△, n=8). The vertical lines show the standard error of the mean.

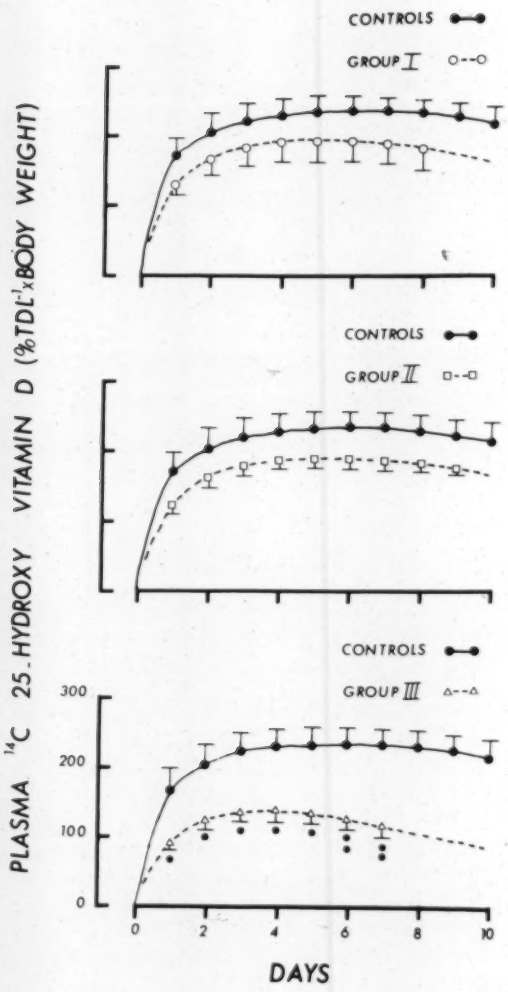

FIG. 2

Concentration-time curves for systemic plasma ^{14}C 25-hydroxy-vitamin D after intraportal injection of ^{14}C vitamin D (per 100 of total dose per litre corrected by body weight -kg-). Comparison in 4 groups of dogs : controls (●—●,n=5), 65 per cent hepatectomized animals (i) without shunt : group I (○- -○, n=8), (ii) with mesocaval shunt : group II (□- -□,n=3), (iii) with portacaval shunt : group III (△- -△,n=8). The vertical lines show the standard error of the mean.

* $p < 0.05$, ** $p < 0.01$.

The time-curves for plasma proaccelerin were similar in groups I and II, and percentages returned to 100 per cent 6 days after hepatectomy (fig.3). On the contrary in group III proaccelerin percentages were still low 14 days after hepatectomy, i.e at the end of the period of biological follow up.

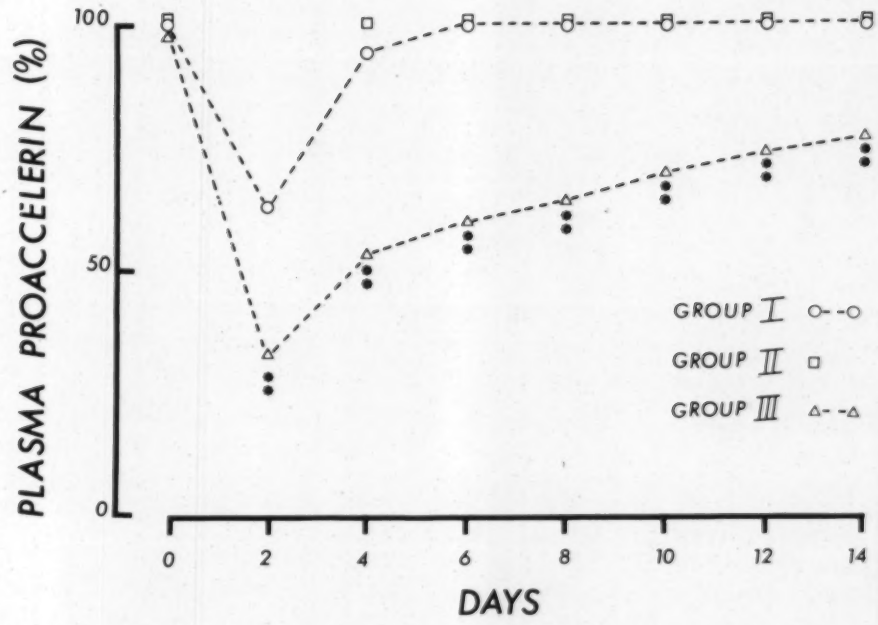

FIG. 3

Time curves for plasma proaccelerin (% of normal). Comparison in 3 groups of hepatectomized dogs (i) without shunt : group I (O--O), (ii) with mesocaval shunt : group II (□--□), (iii) with portacaval shunt : group III (Δ--Δ). Each point represents the mean of the results for 3 to 8 dogs.
● p < 0.01.

DISCUSSION

From the present results, it appears that the plasma kinetics of ^{14}C vitamin D are similar in operated dogs and in controls. As previous studies (17-18) have established that disappearance of ^{14}C vitamin D from plasma is mainly dependent on the amount of vitamin D stored in body tissues, we can conclude that a 65 per cent hepatectomy, with or without portacaval shunt, does not affect the body stores of vitamin D.

According to the results obtained for plasma ^{14}C 25-OH-D activities, vitamin D hydroxylation appears to be unaffected by a 65 % hepatectomy, even when a mesocaval shunt is associated. On the contrary, production of 25-OH-D is markedly altered when the hepatectomy is combined to a portacaval shunt (group III). It is noteworthy that these results are similar to those obtained for proaccelerin.

There was obvious parallelism between changes in 25 hydroxylation of vitamin D and proaccelerin synthesis, and we therefore conclude that both convertion of vitamin D to its 25 hydroxy-derivative and proaccelerin determination provide adequate parameters of liver function .

The results obtained in group I clearly indicate that preservation of 35 per cent of the hepatic mass prevents hepatic insufficiency in the post-operative period, provided that the surgical procedure does not affect the portal blood supply. On the contrary, total diversion of the portal blood into the systemic circulation (group III) results in marked impairment of liver function. Partial diversions such as a mesocaval shunts (group II), which preserve the splenopancreatic blood flow, do not influence remnant liver function.

The differences between groups II and III may be explained by two mechanisms. Firstly, the blood flow to the liver was markedly lower in group III than in II. Secondly, the hormonal secretions of the pancreas in group III were released directly into the systemic circulation, this in turn probably resulting in the reduced availability of pancreatic hormones to the liver. The impairment of liver function observed in group III is probably due to a combination of the two mechanisms. The latter, i.e. the reduction of pancreatic hormone supply to the liver, seems to play a major role since it has been demonstrated that liver trophicity depends on portal hepatotrophic factors (7-14).

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THE EFFECTS OF COMPLEX POLYSACCHARIDES ON GROWTH, DIGESTIBILITY AND BLOOD PARAMETERS IN PAIR-FED CHICKS.

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ABSTRACT

A pair-feeding study was conducted on chicks to evaluate the growth-depressing and hypocholesterolemic effects of guar gum and pectin compared to other sources of dietary fiber. Fibers were fed so that the diet contained 2% hemicellulose or related complex carbohydrate from wheat bran, corn bran, a wood hemicellulose product, guar gum or pectin. A diet containing 10% wheat bran was also fed. Despite pair feeding, final body weights of guar gum-fed chicks were lower than that of all other groups at four weeks. Pectin-fed chicks, though heavier than those fed guar gum, weighed less than those fed the control, wood hemicellulose or wheat bran at either level. Digestibility of the diets appeared to influence growth rates. Metabolizable energy and feed efficiency in guar gum and pectin-fed chicks were also lower, and excreta weight was greater than in chicks fed other sources of fiber. Although not correlated to body weight, total serum cholesterol levels in chicks fed guar gum were lower than those of all other groups. Post-prandial glucose levels in whole blood were highest in chicks fed wood hemicellulose and lowest in those fed guar gum, corn bran or 10% wheat bran.

Key words: guar gum, pectin, cholesterol, hemicellulose, metabolizable energy.

INTRODUCTION

Complex carbohydrates such as guar gum, pectin, cellulose, and hemicellulose are not digested by monogastric animals. These, along with the non-carbohydrate lignin, are called dietary fiber. Carbohydrates found in the indigestible portion of foods vary considerably in chemical structure and physical properties. Therefore, it is not surprising that the effects observed when such carbohydrates are fed to experimental animals are inconsistent. For example, guar gum and pectin fed at a level of 2% in the diet depressed growth in young chickens (1,2) and quail, but not in rats (1). Guar gum was more growth-depressing than pectin. Effects on growth appear to be influenced by other components in the diet besides the dietary fiber source. In one study, growth depression was not observed when either 2% guar gum or pectin was fed in a corn-soybean diet supplemented with cholesterol (3). In other studies, however, in the absence of supplementary cholesterol, a reduction in body weight was observed in chicks fed corn-soybean diets including 2% guar gum, 4% pectin (2,4) or mannan (4). The same level of cellulose did not depress growth when fed as part of a purified casein-glucose or a corn-soybean diet (4).

Other sources of dietary fiber, however, such as wheat bran (5) and Masonex, a hemicellulose-containing product derived from wood (6), were found to be growth-promoting in chicks.

Guar gum, pectin and certain other mucilaginous polysaccharides were shown to be hypocholesterolemic for chicks (3) and rats (7,8) when fed in diets supplemented with cholesterol, but hypocholesterolemic properties varied with changes in dietary protein and carbohydrate sources. In diets composed of casein, gelatin and sucrose, guar gum was more effective in reducing the elevation of sterols and total lipids in the liver of rats caused by cholesterol supplementation than was pectin fed at a dietary level of 10%. However, serum cholesterol was not altered (7). When the diets contained casein instead of gelatin and sucrose at a higher level, pectin appeared to be more effective than guar gum in reducing liver cholesterol and total lipids in rats. Serum cholesterol was unchanged (9). In another study with rats fed casein-sucrose diets supplemented with cholesterol, serum and liver cholesterol levels were reduced, but total carcass cholesterol levels were elevated (10). Hypocholesterolemic properties may also differ with the preparation of the polysaccharide being tested (3).

Diets without cholesterol supplementation have been used in studying the effects of complex carbohydrates on lipid metablism in chickens. Inclusion of pectin or mannan in corn-soy diets resulted in a reduction in liver triglycerides and an elevation of liver cholesterol and phospolipids, but only mannan reduced serum cholesterol (4). Cellulose fed with casein and glucose reduced plasma lipids and liver triglycerides and increased liver diglycerides and phospolipids (4). Patel, et al. (2) observed a decrease in total liver fat and serum cholesterol in chicks fed 4% pectin or 2% guar gum in corn-soybean diets.

Pectin and guar gum are thought to influence lipid metabolism by inhibiting the absorption of cholesterol and increasing its turnover rate in the liver (11). Other types of dietary fiber act differently, and some, like wheat bran, have been found to have little effect on serum lipids (12). In many cases, changes in the level of dietary fat influence the effect of dietary fiber on lipid metabolism (13).

The present experiments were undertaken to study the effects of various complex carbohydrates on serum cholesterol and other parameters when fed as part of an isolated soybean protein-cornstarch based diet containing supplemental cholesterol. To minimize the influence of different dietary intake levels, pair-feeding was utilized in one experiment.

METHODS

Day-old Foster Farms cockerels were weighed, wing-banded and distributed randomly into groups of approximately nine birds. A two-replicate, complete block design was utilized with chicks housed in cages in an electrically heated and temperature controlled metal battery. Each block consisted of one side of the battery unit. Prior to the experiment, the birds were fed a commercial starter diet for two days. All experimental diets (table 1) except the one containing 10% wheat bran were designed to contain 2% complex carbohydrates and 0.5% cholesterol. Therefore, 2.9% corn bran and 2.8% wheat bran were added based on the assumption that they contain 68% and 70% hemicellulose, respectively (14).

TABLE 1.

Composition of Experimental Diets[1]

Ingredient	D-1	D-2	D-3	D-4	D-5	D-6	D-7
				(g/kg diet)			
Basal mix[2]	375.4	375.4	375.4	375.4	375.4	375.4	375.4
Cellulose[3]	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Corn Starch[4]	546.0	526.0	526.0	517.0	518.0	496.0	526.0
Vitamin mix[5]	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Mineral mix[5]	18.6	18.6	18.6	18.6	18.6	18.6	18.6
Guar gum	-----	20.0	-----	-----	-----	-----	-----
Pectin	-----	-----	20.0	-----	-----	-----	-----
Corn Bran[6]	-----	-----	-----	29.0	-----	-----	-----
Wheat Bran[7]	-----	-----	-----	-----	28.0	-----	100.0
Wood Hemicellulose	-----	-----	-----	-----	-----	20.0	-----

1. Diets D-4 and D-5 were designed to contain 2.0% hemicellulose from corn bran and wheat bran. D-2 through D-5 were used in experiment 1 and all diets were used in experiment 2.
2. Basal mix contains (g/kg diet) isolated soybean protein (R P-100, Ralston Purina Co., St. Louis, MO.), 284; $CaHPO_4 \cdot 2H_2O$, 30; $CaCO_3$, 10; dl-methionine, 4.5; NaCl, 4.4; Cholesterol, 5; soybean oil, 37.5.
3. Solka-floc, Brown Company, Berlin, NH.
4. Supplied per kg diet; Vitamin A, 5000 IU; Vitamin D_3, 4500 IU; Vitamin E, 88 IU; Riboflavin, 10 mg; Thiamin HCl 10 mg; Pyridoxine HCl, 10 mg; Menadione, 10 mg; Calcium Pantothenate, 30 mg; Niacin, 120 mg; Folacin, 5 mg; Biotin, 0.4 mg; Vitamin B_{12}, 10 µg; Choline Chloride, 2500 mg; BHT, 1000 mg.
5. Supplied in g/kg diet: $Co(C_2H_3O_2) \cdot 4H_2O$, 0.02; $CuSO_4 \cdot 5H_2O$, 0.097; $FeSO_4 \cdot 7H_2O$, 0.664; KCl, 2.97; K_2HPO_4, 4.95; KIO_3, 0.009; $Na_2SeO_3 \cdot 5H_2O$, 0.00065; ZnO, 0.12.
6. A.E. Staley Manufacturing Co., Dacatur, IL.
7. Masonex, Masonite Corp., Ukiah, CA.

In experiment 1, birds were fed, ad libitum, diets D-2 through D-5 (table 1) containing test materials guar gum, pectin, wheat bran or corn bran; and had free access to water at all times.

In experiment 2, all seven diets in table 1 were used. For the first two days of the experiment, all groups were allowed to eat ad libitum, but the feed consumption was measured. Pair-feeding was initiated on the third day using the group in each block consuming the least amount of food as the control. Initially, the average feed consumption in block 1 was lowest for the corn bran group; in block 2, it was lowest for the guar gum group. By day seven, however, the feed consumption in the guar gum group had fallen behind in block 1, also. For the rest of the experiment, all pair-feeding was done against the guar gum fed groups. Feed was given twice daily at 8:00 and 14:00 for all groups except the guar gum group which ate ad libitum. In both experiments, chicks were weighed twice weekly.

Pooled 24 hour fecal samples were collected on days 19 and 23 of experiment 2 for determination of apparent metabolizeable energy (AME). Gross energy was determined using an adiabatic bomb calorimeter (Parr, model 1241). AME was calculated by subtracting fecal energy from gross energy of feed intake.

Non-fasting blood glucose was measured in whole blood taken from chicks at about 9:30 on days 23 and 26 of experiment 2. Fifty microliters of blood was collected in a capillary pipette from the wing vein. The blood was immediately transferred to a test tube containing 0.95 ml water. After ten minutes, 0.5 ml saturated $Ba(OH)_2$ was added followed by 0.5 ml 0.58 N $ZnSO_4$ after another 10 minutes. Ten minutes later, the samples were centrifuged for 20 minutes at 6500 rpm. Glucose was determined by the glucose oxidase method (15) using 0.5 ml of the supernatant (Kit 115, Sigma, St. Louis, MO).

Chicks were killed at day 30 or 31 in both experiments. Blood samples were drawn by cardiac puncture without anesthetization after an overnight fast. Blood was collected in tubes containing crystalline Na_2EDTA (approximately 25 mg per 5 ml whole blood). After centrifugation, serum was separated and stored at $0^{\circ}C$ until analysis.

Serum triglycerides were determined by the method of Mendez, et al. (16). Total serum cholesterol was determined by a modification of the o-phthalaldehyde method of Zlatkis and Zak after saponification (17). To a 15 ml screw cap test tube were added 0.05 ml serum, 0.15 ml 33% (w/v) KOH (aqueous), and 1.5 ml 95% ethanol. The sample was saponified by heating at $60^{\circ}C$ for 15 minutes in a heating block. After cooling, 5 ml hexane and 1.5 ml distilled water were added. A 3 ml aliquot of the hexane layer was taken and dried under compressed air. To this was added 2.5 ml 30 mg/100 ml o-phthalaldehyde in ethyl acetate[1]. After vigorous mixing to dissolve all dried material, the reaction was completed with 2.5 ml 70% perchloric acid. The final solution was mixed thoroughly and incubated at room temperature for 40 minutes before measuring optical density at 550 nm.

Data from the two experiments were subjected to analysis of covariance ($p<0.05$) using weight as the independent variable, and total serum cholesterol as the dependent variable (18). As no significant regression effects were observed, the data were re-evaluated using one-way or two-way analysis of variance for each variable. In most cases, blocking effects and interactions were insignificant so that one-way ANOVA was appropriate. Differences between cell means were tested using Bonferroni's multiple comparison test at $p<0.05$ (19).

RESULTS

Experiment 1

The data from experiment 1 in which all birds were fed ad libitum are presented in table 2. Mortality was high in the pectin-fed group due to an unexplained loss of all birds in one pen during the first week of the experiment. Chicks fed guar gum or pectin grew poorly compared to those fed corn bran or wheat bran. Serum cholesterol levels were significantly lower in the guar gum-fed chicks than those fed wheat or corn bran, but were not different from those fed pectin. Analysis of covariance indicated no direct relation between body weight and blood cholesterol levels. Serum triglycerides were higher in the pectin-fed group than in the bran groups, but not significantly different than in the guar gum-fed group.

[1] A. Noma, personal communication.

TABLE 2.

Effect of Guar Gum, Pectin, Corn Bran, and Wheat Bran on Body Weight, Serum Cholesterol and Serum Triglycerides of Chickens (experiment 1).

Diet	Treatment	n	Mean Body Wt. (g)	Total Serum Cholesterol (mg/100 ml)	Serum Triglycerides (mg/100 ml)
D 2	Guar gum	9	415.9[a]	102.5[a]	7.85[ab]
D 3	Pectin	7	407.6[a]	164.3[ab]	9.07[b]
D 4	Corn bran	13	697.1[b]	176.7[b]	5.67[a]
D 5	Wheat bran	13	675.5[b]	175.1[b]	5.45[a]
Analysis of Variance					
df treatment			3.0	3.0	3.0
error			38.0	38.0	38.0
MS treatment			249007.88	6866.08	28.349
error			5761.70	1149.66	6.020
F-ratio			43.22	5.97	4.71
Significance (P 0.05)			*	*	*

Analysis of Covariance using serum cholesterol as dependent variable and body weight as independent variable

Source of variation	df	Sum of Squares	Mean Squares	F-ratio	Sign. P<0.05
Equality of adjusted cell means	3	11987.38	3995.79	3.394	*
Zero slope	1	127.36	127.36	0.108	
Error	37	43559.59	1177.29		
Equality of slopes	3	7293.51	2431.17	2.279	
Error	34	36266.08	1066.65		

[a]Different superscripts indicate significant differences between group means within a column.

Experiment 2

Despite assurance that all groups of chicks received equal amounts of food by pair-feeding, final body weights were significantly influenced by the dietary treatments (table 3). The guar gum-fed group had chicks with the lowest body weights. Pectin-fed chicks were significantly heavier than the guar gum-fed chicks, but lighter than all of the other five groups except the corn bran group. Differences in body weight were related to differences in the digestibility of the diets. The apparent metabolizable energy of the diet containing guar gum was significantly less than that of all diets except the one containing pectin. With the exception of the corn bran diet, AME for all other diets were significantly higher than that for pectin. The same pattern was reflected in efficiency of feed conversion to body weight. The feed efficiency was lowest for the guar gum group than for the other six groups which did not differ significantly. The differences in digestibility of the diets were also reflected in excreta weights. Excreta from chicks fed guar gum or pectin weighed about 50% more than excreta from chicks fed the other diets on both wet and dry weight basis.

TABLE 3.

Effect of Dietary Fiber source on Body Weight, Excreta Weight, Apparent Metabolizeable Energy of the Diet (AME), Feed Efficiency, Liver Weight, Serum Cholesterol and Blood Glucose in Pair-fed Chicks (experiment 2). Number of observations per group is given in parenthesis.

Diet	Treatment	Mean body wt. at 4 weeks (g)	wt. of excreta (g/bird/day) wet	wt. of excreta (g/bird/day) dry	AME (Kcal/g)	Feed efficiency (g wt. gain/g feed in)	Liver weight (mg/g body weight)	Total Serum cholesterol (mg/100 ml)	Post-prandial blood glucose (mg/100ml)
D 1	Control	362.1(17)^{a}	15.8(4)^{a}	4.74(4)^{a}	3.628(2)^{a}	0.63(6)^{a}	2.61(16)^{a}	255.1(5)^{a}	273.0(7)^{b}
D 2	Guar gum	218.9(16)^{c}	28.1(4)^{b}	7.69(4)^{b}	2.997(2)^{c}	0.36(6)^{b}	3.42(16)^{b}	121.6(7)^{c}	225.4(8)^{c}
D 3	Pectin	304.4(19)^{b}	24.1(4)^{b}	6.71(4)^{b}	3.185(2)^{bc}	0.50(6)^{a}	2.71(18)^{a}	182.2(6)^{b}	251.3(7)^{b}
D 4	Corn bran	330.4(18)^{ab}	16.7(4)^{a}	4.93(4)^{a}	3.668(2)^{a}	0.57(6)^{a}	2.55(18)^{a}	252.7(6)^{a}	211.8(5)^{c}
D 5	Wheat Bran	362.4(18)^{a}	16.9(4)^{a}	4.72(4)^{a}	3.552(2)^{ab}	0.62(6)^{a}	2.53(18)^{a}	226.2(4)^{ab}	261.2(6)^{b}
D 6	Wood Hemi-cellulose	356.5(17)^{a}	15.2(4)^{a}	4.72(4)^{a}	3.634(2)^{a}	0.64(6)^{a}	2.61(17)^{a}	226.0(8)^{ab}	372.2(4)^{a}
D 7	Wheat bran (10%)	366.1(18)^{a}	15.2(4)^{a}	4.81(4)^{a}	3.645(2)^{a}	0.64(6)^{a}	2.39(16)^{a}	221.8(6)^{ab}	204.4(4)^{c}
Analysis of Variance									
df treatment		6	6	6	6	6	6	6	6
error		116	21	21	7	35	112	35	34
MS treatment		46409.3	104.742	5.5801	144.230	.06553	1.6928	14063.816	14013.474
error		3063.88	26.667	0.4751	11.7313	.01222	0.1313	1281.328	1394.923
F-ratio		15.147	3.928	11.745	12.294	5.3631	12.889	10.976	10.046
Significance(P<0.05)		*	*	*	*	*	*	*	*

Analysis of Covariance using serum cholesterol as dependent variable and body weight as independent variable and body weight as independent variable

Source of Variation	df	sum of squares	mean squares	F-ratio	Significance(P<0.05)
Equality of adjusted cell means	6	60064.63	10010.77	8.014	*
Zero slope	1	1216.34	1216.34	0.974	
Error	35	43720.88	1249.17		
Equality of slopes	6	10076.26	1679.38	1.448	
Error	29	33644.62	1160.16		

[a]Different superscripts indicate significant differences between group means in a column.

In guar gum-fed chicks, the ratio of liver weight to body weight was higher than in the other groups.

Serum cholesterol levels were, as in the first experiment, lower for guar gum-fed chicks than for those fed the other diets. Pectin also reduced serum cholesterol when compared to the control group or the corn bran group. Analysis of covariance again showed no relation between body weight and cholesterol despite the fact that a linear trend could be observed by plotting mean body weights against mean serum cholesterol values for the groups: guar gum, pectin, wheat bran, wood hemicellulose and wheat bran 10%.

Wood hemicellulose increased blood levels after meal feeding, which might have been due to the presence of free glucose in the product. Glucose levels were lowest when corn bran, guar or wheat bran 10% were fed. Since the chicks fed guar gum were fed ad libitum, results from this group must be viewed with caution. Wheat bran at a level of 2.8% did not affect blood sugar levels, but when a higher level of wheat bran was fed, blood sugar levels were lower than that of controls.

DISCUSSION

The results of this experiment clearly indicate that poor growth in chicks fed guar gum or pectin was due to a decrease in the digestibility of the diet when these complex carbohydrates were present at a relatively low level. Although guar gum-fed chicks ate less than normal, their weight gains were still lower than those of chicks fed equivalent amounts of diets containing different types of complex carbohydrates. Chicks fed guar gum appeared unkempt. The vent area was swollen and feathers were sparse. The diet pasted around the beak making eating sufficient quantities impossible. Pectin diets, though less problematic, also caked on the beak.

Although a decrease in the apparent digestibility of most components of a high fiber diet containing wheat bran was observed in humans by Farrell, et al. (20), the authors observed no decrease in AME for chicks fed wheat bran at either 2.8 or 10%.

The weight of excreta is greatly increased in chicks fed guar gum or pectin diets (Table 3). A larger amount of water was present, but dry matter was also increased. However, no differences in excreta weight were observed between chicks for 2.8% and 10% wheat bran diets. Gross energy of all diets were similar (4.10-4.20 kcal/g), but AME was significantly lower for the guar gum and pectin groups. Digestibility of the diet and, thus, the availability of nutrients including cholesterol and fat, is decreased in these diets more than in the others. This was previously observed by Kratzer, et al. (21) who found that a 2% level of guar gum fed to chicks reduced absorption of fat and nitrogen retention. They did not, however, observe the same results when pectin was fed.

It is difficult to compare different sources of dietary fiber between experiments or within a single experiment especially when the absolute composition of the fiber is unknown. Comparison of different fibers for metabolic effects is also difficult because several mechanisms may be responsible for the same clinical properties.

Dietary fibers are mainly complex carbohydrates such as cellulose, pectin, gums and hemicellulose bound in the plant cell wall to each other or to proteins or lignin. The structures of guar gum and pectin are well

established. Guar gum is a galactomannan derived from the legume, Cyanopsis tetragonoloba. Pectin is a methoxylated derivative of polygalacturonic acid. The wood hemicellulose product used in this experiment was a by-product of the production of hardwoods, and contained a high content of phenolic compounds and free sugars (6). Preliminary analysis by gas chromatography indicates that free glucose is present in significant amounts. The phenolic compounds present are probably derived from lignin. Though no effects were seen in the present study by feeding wood hemicellulose, some investigators have reported that lignin will reduce blood cholesterol levels in rats (21). This property appears to be dependent on the lignin source, however (23). Alteration in the lignin structure during processing as well as the type of wood used might affect the ability of the wood hemicellulose product to reduce cholesterol levels.

The hypocholesterolemic and growth-depressing properties of certain polysaccharides depend not only on their chemical formulas, but also on the physical form and particle size (24). Guar gum and pectin are mucilaginous compounds with large water-holding capacities. Wood hemicellulose, a syrupy liquid, is soluble in water and can be diluted without forming a gel. Wheat bran and corn bran have a different structure, and although they can absorb water, they do not form gels. Pectin and guar gum have the capacity to sequester nutrients such as fat, bile acids and cholesterol within the matrix of the gel; brans adsorb compounds to their surface. Wood hemicellulose would be expected to have neither of these properties.

Earlier studies imply that wood hemicellulose does not interfere with absorption and digestion, but enhances the feed intake and weight gain in growing chicks (6). The growth-enhancing properties of Masonex were attributed to the presence of high molecular weight phenolic compounds. Increased feed intake was thought to be related to a factor different from that which caused the increased growth. In the present study, however, the addition of wood hemicellulose to the diet did not improve growth above that of the control when food intake was equalized by pair feeding.

Absorption of bile acids is independent of the water-holding properties of bran. Pectin is more effective in depressing cholesterol levels when it is present as a gel rather than as a powder. Some reports indicate that the pectins with a higher methoxy content have greater hypocholesterolemic properties, but others believe that it is the viscosity of the pectin which causes lowering of cholesterol levels (22).

A number of studies have focused on the effects of dietary fiber on glucose metabolism. High fiber diets have been advocated for the treatment of diabetes mellitus in man as a means of lowering blood glucose levels (25). Guar gum and pectin have been shown to lower post-prandial blood glucose levels in both normal and insulin-dependent diabetics (25,26). Fiber is thought to have its effect on blood glucose by slowing the rate of gastric emptying (27). The effects of a fiber on glucose metabolism may be related to its effect on lipid metabolism. It has been shown that insulin increased cholesterol synthesis and VLDL secretion from the liver (24). It is not unexpected, then, that mucilaginous carbohydrates which are most effective in depressing plasma cholesterol levels were also the ones that maintained a lower post-prandial glucose profile. In the present study, however, one cannot predict post-prandial glucose levels on the basis of blood cholesterol. Glucose levels were lowest in chicks fed guar gum, corn bran or high levels of wheat bran. Glucose levels were higher in pectin-fed chicks, but were highest when wood hemicellulose was fed. Though the guar gum-fed group had the lowest cholesterol levels, and though their blood glucose levels might be expected to be low because they were fed ad libitum as opposed to meal feeding, their glucose levels were not significantly less than those of chicks fed corn bran or 10% wheat bran.

It is interesting that there were no differences between the 2.8% and 10% wheat bran groups in any of the variables studied except for blood glucose levels. With the higher level of wheat bran, blood glucose was reduced. Increased fecal weights in studies where wheat bran was fed at high levels could be related to greater intakes of reduced caloric density diets rather than increased bulkiness caused by greater uptake of water by bran. With only a few exceptions, the inability of wheat bran to decrese serum cholesterol levels has been well documented (11). Farrell, et al. (20), using men as their own controls, found lowered cholesterol levels when high bran diets were eaten. Munoz, et al. (26), observed cholesterol-lowering effects in man with hard spring wheat but not with the soft wheat most frequently used in feeding studies.

In the present study, significantly different liver to body weight ratios were observed, only in the group fed guar gum. The increased proportion of liver weight may have been due to diminished body fat depots in these chicks. It appears that poor utilization of guar gum in chicks is related to an inability to catabolize the galactomannan to its constituent sugars. Growth depression was alleviated in chicks fed guar gum (2, 29) or pectin (2, 30) that had been first treated with hydrolytic enzymes.

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ADAPTATION OF RAT PARENCHYMAL HEPATOCYTE TO NUTRITIONAL VARIATION: QUANTITATION BY STEREOLOGY[1]

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ABSTRACT

The response of parenchymal hepatocytes to nutritional variation was quantitated using stereological analysis of electron and light micrographs. Young male rats (150 g) were acclimatized for four days with stock diet and then randomized by weight into three groups. After a 48-hour fast, rats were offered ad libitum access to a high carbohydrate/fat-free diet (Diet 1), a low carbohydrate/protein-free diet (Diet 2), or stock diet. Rats were sacrificed after five days of feeding. Cellular volumes of C-3,4 hepatocytes (3rd and 4th cells from the central vein) were 4400, 6500 and 2600 μm^3 for rats fed stock diet, Diet 1, or Diet 2, respectively. Nuclear volumes from these cells were 230, 370 and 250 μm^3, respectively. Compared to control diet, Diet 1 significantly increased fractional volumes of cytoplasm occupied by mitochondria, glycogen and lipid and increased surface density of rough endoplasmic reticulum (RER). Diet 2, compared to Diet 1 and control diet, elevated volume fractions of glycogen, lipids and lysosomes and decreased cytoplasmic volume fractions of mitochondria and peroxisomes and surface densities of RER and Golgi cisternae. Absolute volumes of various organelles, membrane areas for RER, smooth endoplasmic reticulum, and outer nuclear envelope, and number of mitochondria, peroxisomes and lysosomes were quantitated for C-3,4 hepatocytes under each nutritional influence.

KEY WORDS: Parenchymal hepatocyte, protein-free, fat-free, stereology

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INTRODUCTION

The parenchymal hepatocyte, due to its site and multi-functional nature, is responsive to many fluctuations in its near environment. Nutritional intake alters the histochemistry (1,2), biochemistry (3-6), genetic machinery (7-10), and ultrastructure of the parenchymal hepatocyte (8,11). Ultrastructure analyses, in the past, have been characterized by limited quantitation. The development and application of stereological techniques (12,13) has allowed the quantitative estimation of fractional component volumes and surface areas, mean particle volume and surface areas, as well as particle number and size (14,15).

Stereology is a technique used to derive three-dimensional information from two-dimensional data. It is based upon the principle that the fractional volume of a component is equal to its fractional area in a random cross-section of negligible thickness (16). Stereological examination of morphometric properties of the normal parenchymal hepatocyte has been used to quantitate the fractional volumes of cytoplasm occupied by mitochondria, peroxisomes, lysosomes, lipid, and glycogen (12,13).

Previously, we demonstrated (7) that nutritional intake alters the organization of rat liver chromatin. Two nutritional stimuli, in particular, a high carbohydrate/fat-free or a low carbohydrate/protein-free diet (hereafter referred to as Diet 1 and Diet 2, respectively), modulated the total amount of chromatin susceptible to a hydrolytic nuclease. We now quantitate by stereological analysis the cellular and subcellular alterations attributed to these same dietary treatments.

MATERIALS AND METHODS

Experimental Animals. Young male Holtzman rats weighing approximately 150 g were housed individually in suspended wire-mesh cages. Rats were fed stock diet (Ralston Purina Company, St. Louis, MO) for a four-day acclimatization period then randomized by weight and allotted to one of three groups in a randomized block design. After a 48-hour fast, rats were offered ad libitum access to water and feed beginning at 0900. At 0900 on the fifth day of feeding, rats were sacrificed by cervical dislocation. Sacrifice schedule proceeded in blocks to eliminate diurnal variation.

Diets. Compositions of the experimental diets are presented in Table 1. Detailed descriptions of the salt mixture and vitamin mixture have been described previously (7).

Tissue Preparation. Immediately after sacrifice, the liver was resected in toto and weighed. Tissue samples were taken from the left lateral lobes, submerged in 1.5% glutaraldehyde - 0.2 M cacodylate buffer (pH 7.2) and diced into 1 mm cubes. The time from sacrifice to glutaraldehyde fixation was less than 30 seconds. The cubes were transferred to fresh glutaraldehyde and fixation was continued at 4° for 20 hours. The samples were postfixed in 1% OsO_4 for 2 hours at 4°, dehydrated in an ascending ethanol series, and embedded in Epon (17). Three blocks from each animal were randomly sectioned for both light and electron microscopy. For light microscopy, 1 µm sections containing a central vein were stained with toluidine blue and photographed at a final magnification of 790 X. These micrographs were used to determine cell size and nuclear diameter of hepatic parenchymal cells.

TABLE 1

Composition of Diets (g/100 g)

Component	Diet 1 High carbohydrate/ fat-free	Diet 2 Low carbohydrate/ protein-free
Casein, vitamin-free[1]	30	0
D(+)- Dextrose[1]	60	18
Salt mixture[1]	4	4
Alphacel (non-nutritive bulk)[1]	4	67
Vitamin mixture[1]	2	2
Corn Oil[2]	0	15

[1]ICN Nutritional Biochemicals, Cleveland, OH

[2]CPC International Inc., Englewood Cliffs, NJ

Thin sections, ranging in thickness from 600 to 900 Å were obtained adjacent to the above thick sections and stained with uranyl acetate and lead citrate. Adjacent, but nonoverlapping micrographs were randomly taken of the 3rd and 4th parenchymal cells from central veins. These cells will be referred to as C-3,4 cells. The micrographs were examined at final magnifications of 16,000 and 48,000 X using a lattice grid for point and linear analysis. The magnifications were calibrated using a carbon grating having 2,160 lines/mm and the magnification variation was less than 2% throughout this study. The experimental design for electron microscopy consisted of three dietary treatments with 3 animals/treatment; 2 blocks were evaluated from each animal and 6 micrographs were evaluated at 16,000 X and 6 micrographs at 48,000 X for each block.

Stereological Methods. The stereological methods used to quantitatively evaluate the sectioned liver tissue have been previously described by Loud (12), Weibel et al. (13) and Weibel and Bolender (14). Volume fractions (V_V) were determined by point analysis. These data were expressed as volume per volume, e.g., the volume (or per cent) of mitochondria per volume of cytoplasm. The grid size was selected so one point, on average, would fall on each of the component profiles being examined. The relative standard error (18) was less than 5% for each component examined.

Surface density, S_V, which is the area of a membrane in a test unit volume, was calculated directly from counts of the intersection points I_i of the surface contour of profiles with test lines of known length L_t by the following equation (19):

$$S_V = \frac{2I_i}{L_t}$$

The average dimensions of the hepatic parenchymal cells (C-3,4 hepatocytes) from rats fed the 3 different diets were determined from 85 light

micrographs. The average diameter of C-3,4 hepatocyte nuclei for each treatment was determined by measuring the diameter of 583 nuclear profiles ($\bar{d}$) and estimating the mean nuclear diameter ($\bar{D}$) by Abercrombie's (20) formula:

$$\bar{D} = \bar{d} \times 4/\pi$$

The area of parenchymal cells in the light micrographs was measured by point counting. The number of nuclei within this area (N_A) was used to calculate the number of nuclei per unit volume (N_V) by:

$$N_V = N_A/(\bar{D} - 2p + t)$$

where t represents section thickness and p is a consideration of the distance a particle must penetrate into a section before it is visible. The value for p of 0.3 μm for nuclear N_V (12) was used.

The reciprocal of N_V, parenchymal cell volume per nucleus, minus the volume of an average nucleus represents the average volume of cytoplasm (μm^3) per cell (nucleus). This value was used to calculate the absolute values of components per cell (nucleus) from the quantitative electron microscopic data.

For computation of numerical densities of mitochondria, peroxisomes and lysosome-like bodies we used the following equation (14):

$$N_V = \frac{K}{\beta} \cdot \frac{N_A^{3/2}}{V_V^{1/2}}$$

Peroxisomes and lysosome-like bodies were presumed spherical and 1.38 was used for the value of β. To determine the value of β depending on the axial ratio (λ) of cylinders, we measured the axes of 100 randomly selected mitochondria from each animal; and the corresponding value of β was read from the diagram of Weibel and Bolender (14). The β values were 1.87, 1.87 and 1.77 for mitochondria from animals fed the stock diet, Diet 1 and Diet 2, respectively. As suggested by Weibel and Bolender (14), K was disregarded as it is virtually 1.

The means of the various measurements were analyzed using Duncan's multiple range test (21) at the 0.05 probability level. The use of the term significant in this study will infer the 95% confidence level.

Systematic errors were not corrected, as the results are equally influenced and their effect can be negated in comparisons (13). We did take into account the intralobular localization of the liver cells by examining only C-3,4 hepatocytes since Loud (12) showed considerable heterogeneity between parenchymal cells depending upon their intralobular position.

RESULTS

Animal Growth. Weight parameters as well as food and caloric intake are presented in Table 2. The average initial body weights varied less than 1 g among all three groups. The average daily food and caloric intakes varied appreciably and were reflected in the wide range of final body weights.

Light Microscopy. Nuclear and cytoplasmic volumes (μm^3) are presented in Table 3. The total cytoplasmic volume of C-3,4 hepatocytes from rats fed Diet 2 was only 40% that from rats fed Diet 1, and 59% that from rats fed

TABLE 2

Body and Liver Weights, Food Intake and Caloric Intake of Rats Fed Stock, Lipogenic, or Low Carbohydrate/Protein-Free Diets

Diet	Number of animals	Initial body wt (g)	Final body wt (g)	Liver wt (g)	Food intake (g/day/animal)	Caloric intake (kcal/day/animal)
Stock	3	181.8 ± 10.4[1]	206.5 ± 12.2	6.8 ± 1.9	20.7 ± 1.8	87.9 ± 7.6
Diet 1	3	182.7 ± 7.0	192.2 ± 8.3	10.0 ± 1.0	17.0 ± 2.1	62.7 ± 7.6
Diet 2	3	182.1 ± 6.9	148.2 ± 3.3	4.3 ± 1.5	16.0 ± 2.2	34.3 ± 4.6

[1]Mean ± S.D.

TABLE 3

Nuclear and Cytoplasmic Volumes of C-3,4 Hepatocytes

Diet	N (Number of Nuclei Measured)	$\bar{D}_n$ (Mean Nuclear Diameter)	Cell Area Measured (μm^2)	N_A (Number of Nuclei/$10^4 \mu m^2$)	N_V (Number of Nuclei/$10^6 \mu m^3$)	Average Nuclear Volume (μm^3)	Average Cytoplasmic Volume (μm^3)
Stock	100	7.56 b	58,804	17.0	214	226	4447
Diet 1	148	8.89 a	109,758	13.5	145	368	6529
Diet 2	335	7.85 b	117,008	28.6	347	253	2629

*Means within a column followed by the same letter were not different at the 0.05 probability level using Duncan's multiple range test.

stock diet. In contrast, total cytoplasmic volume of hepatocytes of rats fed Diet 1 was 47% larger than that of hepatocytes from stock-fed rats. The cellular hypertrophy exhibited by these hepatocytes from Diet 1 was also reflected by a larger nuclear volume and a larger mean nuclear diameter.

The ratio of observed binucleate to uninucleate cells was 0.04, 0.03 and 0.14, respectively, for the stock diet, Diet 1 and Diet 2. For hepatocytes of rats fed Diet 2, the decreased cytoplasmic volume without a concommitant decrease in mean nuclear diameter provided a section geometry that would explain the apparent increase in the binucleate/uninucleate ratio. Binucleate cells appear to have twice the volume of mononucleate cells (22), therefore, the average volume of cytoplasm per cell (nucleus) would be expected to closely approximate the average uninucleate cell (12). In this study, the electron microscopic quantitative data are reported as the value of components present per cell (nucleus).

Electron Microscopy

The various parameters determined in this stereological assessment of hepatic parenchymal cells from rats fed different diets are presented in Tables 4-7. The volume fractions (V_V) of several cytoplasmic components are shown in Table 4. The mitochondrial V_V of C-3,4 hepatocytes from Diet 2-fed rats was 10% less than that of hepatocytes from stock-fed rats and was 22% less than that from Diet 1-fed rats. In contrast, glycogen V_V of C-3,4 hepatocytes from rats fed Diet 2 was 56% and 25% greater than that of hepatocytes from rats fed stock diet or Diet 1, respectively. Lipid V_V of C-3,4 hepatocytes from rats fed Diet 2 was 2.4 times and 11 times greater that from Diet 1- and stock-fed rats, respectively. Peroxisome V_V of C-3,4 hepatocytes from rats fed Diet 2 was 60% that from stock-fed rats, but quantitatively similar to that from Diet 1-fed rats. Lysosomal V_V of C-3,4 hepatocytes from Diet 2-fed rats was approximately twice that of hepatocytes from Diet 1- and stock-fed rats. Between the latter two there was no difference between lysosomal V_V. The fraction volume occupied by Golgi cisternae in C-3,4 hepatocytes of Diet 2-fed rats was less than half that in hepatocytes from rats fed stock diet or Diet 1.

Table 5 shows that the area of RER membranes per cytoplasmic volume in hepatocytes of rats fed Diet 1 was 26% and 43% greater than that in hepatocytes of rats fed stock diet or Diet 2, respectively. No difference among any of the diets was discerned for the areas of the smooth endoplasmic reticulum (SER) and nuclear envelope per unit cytoplasmic volume. The area of outer mitochondrial membrane per unit cytoplasmic volume (Table 5) and and per unit mitochondrial volume (Table 6) was significantly lower in hepatocytes of rats fed Diets 1 and 2, compared with that from stock-fed rats. In contrast, no difference was observed among diets for the area of cristae per mitochondrial volume (Table 6).

Actual volumes and areas of various entities on a per cell basis are presented in Table 7. In C-3,4 hepatocytes of rats fed Diet 1, the larger volumes for glycogen, peroxisomes, lysosome-like bodies, Golgi cisternae, lipid, and nucleus compared with the other two groups reflect overall larger cell size. The increased mitochondrial volume calculated per average cell (nucleus) in hepatocytes of rats fed Diet 1 was due to an actual increased mitochondrial volume as seen when calculated as per cent of cytoplasmic volume (Table 4).

The membrane area of RER, SER, and outer nuclear envelope are calculated on a per cell (nucleus) and also are shown in Table 7. The area of each of these three membranes in hepatocytes of rats fed Diet 1 was greater than those in heptocytes of the rats in the two other groups. Areas of all three

TABLE 4

Volume Fractions (V_v) of Cytoplasmic Components of C-3,4 Hepatocytes

(% of Cytoplasmic Volume)

Diet	Mitochondria	Glycogen	Peroxisomes	Lysosomes	Lipid	Golgi Cisternae
Stock	15.3 b	13.7 c	1.09 a	0.54 b	0.31 b	0.78 a
Diet 1	17.5 a	17.1 b	0.97 ab	0.61 b	1.46 b	0.82 a
Diet 2	13.7 c	21.4 a	0.67 b	1.21 a	3.47 a	0.34 b

*Means within a column followed by the same letter were not different at the 0.05 probability level using Duncan's multiple range test.

TABLE 5

Membrane Areas/Cytoplasmic Volume ($\mu m^2/\mu m^3$)[1] of C-3,4 Hepatocytes

(% Cytoplasmic Volume)

Diet	RER[2]	SER[3]	Nuclear Envelope	Mitochondrial Outer Membrane
Stock	2.77 b	1.42 a	0.28 a	2.66 a
Diet 1	3.48 a	1.31 a	0.28 a	2.32 b
Diet 2	2.43 b	1.27 a	0.21 a	1.98 b

[1]Values are the sum of both membrane surfaces. Means within a column followed by the same letter were not different at the 0.05 probability level using Duncan's multiple range test.

[2]Rough endoplasmic reticulum

[3]Smooth endoplasmic reticulum

TABLE 6

Membrane Areas/Mitochondrial Volume ($\mu m^2/\mu m^3$)* of C-3,4 Hepatocytes

(% Mitochondrial Volume)

Diet	Mitochondrial Outer Membrane	Mitochondrial Cristae
Stock	8.9 a	22.8 a
Diet 1	6.9 b	23.0 a
Diet 2	7.3 b	25.4 a

*Values are the sum of both membrane surfaces. Means within a column followed by the same letter were not different at the 0.05 probability level using Duncan's multiple range test.

membranes were smallest in hepatocytes of rats fed Diet 2. The membrane area of rats fed Diet 1 was 84% greater than that of stock-fed rats, whereas the RER membrane area of Diet 2-fed rats was 48% less than that of stock-fed rats.

TABLE 7

Ultrastructural Composition of C-3,4 Parenchymal Hepatocytes Calculated Per Average Cell (nucleus)

	Diet		
Volume (μm^3)	Stock	1	2
Total Cytoplasm	4447	6529	2629
Mitochondria	680	1143	360
Glycogen	609	1116	563
Peroxisomes	48	63	18
Lysosome-Like Bodies	24	40	32
Golgi Cisternae	35	54	9
Lipid	14	95	91
Nuclei	226	368	253
Membrane Area (μm^2)			
RER[1]	18500	34100	9600
SER[2]	9500	12800	5000
Outer NE[3]	1900	2700	800
Number			
Mitochondria	2146	1677	783
Peroxisomes	204	451	105
Lysosome-Like Bodies	342	886	278

[1]Rough endoplasmic reticulum

[2]Smooth endoplasmic reticulum

[3]Nuclear envelope

The actual number of mitochondria, peroxisomes, and lysosome-like bodies also are presented in Table 7. The C-3,4 hepatocytes of Diet 2-fed rats contained only 36%, 51%, and 81% of the number of mitochondria, peroxisomes,

and lysosome-like bodies, respectively, as in hepatocytes of stock-fed control rats. Hepatocytes of rats fed Diet 1 contained only 78% the number of the mitochondria, but greater than twice the number of peroxisomes and lysosome-like bodies in hepatocytes from rats fed stock diet.

DISCUSSION

The response of the parenchymal hepatocyte to its nutritional environment has been quantitated by stereological analysis. When the cytoplasmic volume of stock-fed rats was taken as the norm, hypertrophy of the parenchymal hepatocyte was evident in liver of rats fed Diet 1, whereas atrophy of the parenchymal hepatocyte was exhibited in liver of rats fed Diet 2. Svoboda and Higginson (11) qualitatively observe decreased cell size in liver of rats fed a protein-free diet ad libitum or force-fed 4% casein.

The surface area of endoplasmic reticulum was expressed per cytoplasmic volume and per average cell (nucleus). For all three dietary treatments the S_V of SER was similar. The S_V of RER for rats fed Diet 1 was greater than those for rats fed stock diet or Diet 2. A major function of the liver is the synthesis and secretion of plasma proteins. Polysomes bound to RER (23) are the sites at which such serum proteins as albumin, glycoproteins, fibrinogen, and prothrombin (24) are synthesized. The surface density, S_V, of the RER was significantly reduced in hepatocytes from Diet 2-fed rats compared to Diet 1-fed rats. Likewise, there was a marked reduction in the Golgi cisternae in the hepatocytes from the rats fed Diet 2, suggesting a decreased secretory activity. The protein synthetic and secretory activity appeared to be enhanced in hepatocytes from animals fed Diet 1. Increased protein synthesis in hepatocytes from rats fed Diet 1 is supported by two-dimensional gel electrophoresis which reveals a greater number of proteins being synthesized in liver of rats fed Diet 1 compared with Diet 2 (25).

Mitochondrial V_V, the area of mitochondrial outer membrane to mitochondrial volume and to cytoplasmic volume, and the volume of mitochondria per average cell (nucleus) were decreased in hepatocytes from rats fed the protein-free diet (Diet 2) as compared with hepatocytes from stock-fed rats. Occurring primarily in the mitochondrion is fatty acid oxidation, which is quantitatively the most important source of energy for hepatic metabolic needs (24). The observed atrophy of mitochondria presumably reflects depressed levels of mitochondrial respiratory functioning.

When the fractional volume (V_V) of lipid was measured and calculated as percentage of cytoplasmic volume, lipid V_V in hepatocytes of Diet 2-fed rats was 11-fold and two-fold greater than that in rats fed stock diet or Diet 1, respectively. Fatty acids may have accumulated in the cell due to decreased mitochondrial oxidation of fatty acids in hepatocytes of rats fed Diet 2. The majority of the fatty acids may become esterified with α-glycerophosphate resulting in the accumulation of triacylglycerides (24). Because of an apparent absence of feedback inhibition in the synthesis of triacylglycerides, they accumulate progressively, even when the need is lacking (24). Svoboda and Higginson (11) qualitatively observe slight fat accumulation in rats after 10 days of feeding a protein-free diet.

In addition to an increased lipid V_V, there was a significant increased glycogen V_V for Diet 2-fed rats. Marked accumulation of glycogen was observed in young rats fed ad libitum a 4% casein diet (20). The cause for increased glycogen may be explained in terms of amino acid metabolism. Simultaneous calorie and protein restriction results in a rise of nonessential amino acids

and a decreased ratio of essential:nonessential amino acids (26). Many of the nonessential amino acids being glucogenic may explain the accumulated glycogen volume in rats fed a low carbohydrate/protein-free diet.

In summary, the morphometric responses of parenchymal hepatocytes to a high carbohydrate/fat-free diet, a low carbohydrate/protein-free diet, or stock diet have been quantitated by stereology. Cellular and nuclear volumes are presented as well as volume fractions of subcellular organelles and surface densities of membranes.

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DISTRIBUTION OF EXOGENOUS RADIOLABELED CHOLESTEROL IN RAT INTESTINE: EFFECT OF DIETARY FIBER

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ABSTRACT

The distribution of radioactive neutral and acidic steroids in rat intestine was studied following oral administration of 4-^{14}C-cholesterol. The influence of dietary fiber was examined in three dietary groups: a commercial ration; a semipurified, fiber-free diet; and the latter diet with cellulose added. The amount of radioactivity remaining in the intestinal tissue and contents 3 days after isotope administration equaled that found in the serum plus liver pool. The distribution of cholesterol-associated radioactivity in the intestines indicated probable incorporation into epithelial cell membranes of the small bowel. Dietary fiber significantly influenced the concentration of radioactive steroids primarily in the large intestine.

KEY WORDS: Cholesterol, cellulose, diet, intestinal absorption, dietary fiber, rats

INTRODUCTION

The relatively low recovery of radioactively-labeled cholesterol in serum, liver and feces of rats following oral administration of tracer doses suggests that significant amounts of exogenous cholesterol are temporarily retained in some body pool, possibly in the gastrointestinal (GI) tract. Borgström et al. (1) concluded that the delayed appearance of cholesterol-associated radioactivity in blood was caused by the retention of newly absorbed cholesterol in the intestinal wall and later in the liver before its release into the circulation.

The steroid content of the GI tract is influenced by type and amount of dietary fiber. Portman (2) reported that rats fed semipurified (SP) diets containing either no fiber or cellulose, excreted much less cholic acid and had decreased bile acid turnover compared to rats fed commercial laboratory ration. Sterol excretion occurs primarily via direct loss of fecal neutral steroids or bile acids (3,4). The distribution of acidic steroids along the small intestine has been related to the type and concentration of non-digestible carbohydrate (5). However, quantitation of bile acids in both small and large intestine as a function of dietary fiber has not, to our knowledge, been studied. This report details our studies of the contribution of the GI tract in temporary sequestration of steroids as a function of diet.

MATERIALS AND METHODS

Twenty-four male Wistar rats were derived into three equal groups with an average weight of 197 grams. One group was fed a commercial ration (Ralston Purina Co., St. Louis, Mo.). The second received a semipurified (SP) diet composed of 45 parts sucrose, 25 parts casein, 14 parts corn oil, 1 part ICN vitamin fortification mix, and 5 parts salt mix USP XIV supplemented with zinc and chromium. The third group was given the SP diet to which 10 parts cellulose were added (SPC). The animals were fed ad libitum for 14 days and each was given a mixture of 0.5 μCi of 4-^{14}C-cholesterol (New England Nuclear, Boston, Ma.) plus 1 mg of unlabelled cholesterol in 0.5 ml propylene glycol by stomach tube. The rats were placed in cages containing raised stainless steel wire floors and the feces were collected for 3 days.

The rats were killed by decapitation, blood was collected, and the livers and GI tracts were excised. The GI tract was separated into four segments: stomach and proximal half of the small intestine, distal half of the small bowel, cecum, and the colon. Intestinal contents were carefully removed using cold saline. The contents and the GI tissue were saponified separately with 15% KOH in 50% ethanol at 37°C for 48 hours. Liver and serum lipids were extracted with chloroform:methanol (2:1) and sterols precipitated as the digitonides. Cholesterol of serum and liver was quantitated using an enzymatic assay (6,7). Extraction of fecal neutral and acidic steroids was performed as previously described (8). The steroids were isolated, dried and assayed for radioactivity in a Beckman liquid scintillation spectrometer with a counting efficiency of 91.9% for ^{14}C. Data are expressed as mean + standard error of the mean. Statistical comparisons were performed using analysis of variance followed by the Newman-Keuls multiple range test (9).

RESULTS

In all three groups, the amount of radioactive steroid remaining in the intestines (both tissue and contents) 3 days after oral administration of 4-^{14}C-cholesterol was similar to the radioactivity found in the serum and liver pool (see Table 1).

Only negligible amounts of labeled cholesterol were found in the contents of the small intestine (Figure 1a). In contrast, both the cecal and colonic contents contained significant amounts of labeled steroid and in some cases recovery equaled the radioactivity calculated for the plasma compartment. The labeled cholesterol concentration of the cecal tissue was influenced significantly by the diets. Rats fed the stock diet showed the lowest level of radioactivity in neutral steroids and rats given the fiber-free SP ration had significantly more radioactivity. Radiolabeled acidic steroid found in the lumenal contents of the small intestine was low (0.1 - 0.2% of original dose) and may represent bile that was recycled in the enterohepatic circulation (Figure 1b). The highest concentrations of radioactivity found in the intestinal samples were those of the acidic steroid fractions of the contents from the large intestine. Rats fed the commercial diet had the highest amount of labeling in the cecum which was significantly greater than that observed in the SPC group. The acidic colonic contents were labeled to a significantly greater degree in the stock diet-fed animals than in either of the other two groups.

The radioactivity associated with the intestinal tissue segments presumably represents sterol either in transit through the wall or that which has been incorporated into cell membranes for structural purposes. Substantial amounts of tissue-associated, labeled cholesterol were detected in both the proximal and distal portions of the small intestine (Figure 2a). Radioactivity associated with neutral sterol of the cecal wall differed as a

TABLE 1

Serum and Liver Cholesterol Levels and Radioactivity (CPM x 10^{-4}) of Steroids Isolated from Serum, Liver and Intestine of Rats Fed Three Diets

	Stock	SP	SPC
Cholesterol			
Serum (mg/100 ml)	91 ± 3	102 ± 7	91 ± 6
Liver (mg/100 g)	359 ± 21[a]	399 ± 26	471 ± 24[a]
Radioactivity			
Serum	1.56 ± 0.11	1.44 ± 0.10	1.27 ± 0.11
Liver (neutral)	4.85 ± 0.31[b,c]	6.25 ± 0.40[b]	6.19 ± 0.38[c]
(acidic)	0.91 ± 0.08	1.20 ± 0.05	1.29 ± 0.05
Serum + Liver pool	7.31 ± 0.48	8.88 ± 0.42	8.74 ± 0.47
Intestines	9.23 ± 0.49	8.80 ± 0.55	7.44 ± 0.62

Numbers with same superscripts are significantly different by Newman-Keuls multiple range test ($p < 0.05$).

TABLE 2

Fecal Radioactivity in Neutral and Acidic Steroids of Rats Fed Three Diets

	Stock	SP	SPC
Fecal dry wt (g)	7.6 ± 0.4[a]	3.0 ± 0.4[a]	5.3 ± 0.6[a]
CPM x 10^{-4}/GM			
Neutral	2.52 ± 0.26[b]	10.57 ± 1.13[b]	5.26 ± 0.47[b]
Acidic	0.81 ± 0.16[c,d]	1.78 ± 0.19[c]	2.20 ± 0.42[d]
TOTAL CPM x 10^{-5}			
Neutral	1.94 ± 0.27[e]	3.15 ± 0.46[e]	2.80 ± 0.40
Acidic	0.62 ± 0.13	0.51 ± 0.05[f]	1.05 ± 0.16[f]
Total	2.57 ± 0.32[g,h]	3.66 ± 0.50[g]	3.85 ± 0.32[h]

Numbers with the same superscripts are significantly different by Newman-Keuls multiple range test ($p < 0.05$).

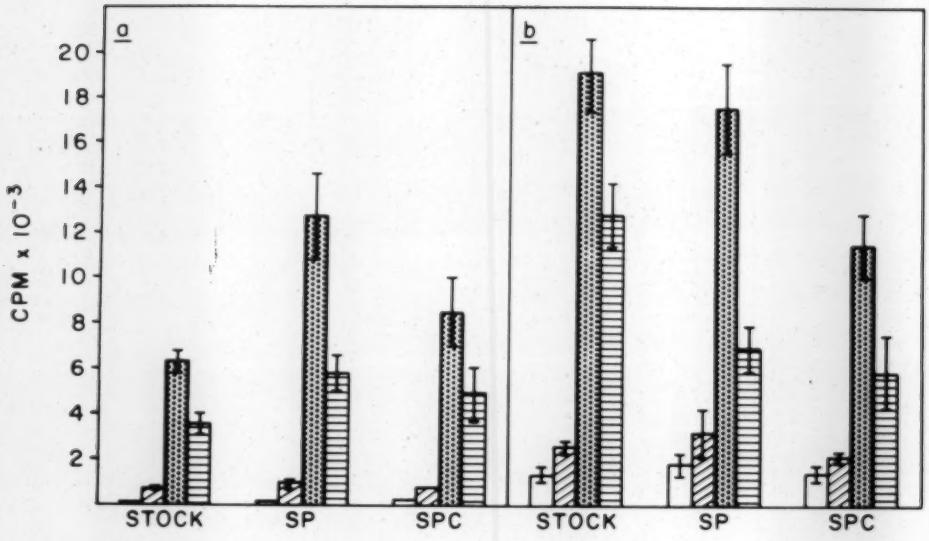

FIG 1

Radioactivity recovered from intestinal contents associated with neutral steroids (1a) and acidic steroids (1b). Open columns, stomach and proximal half of small intestine; diagonally lined column, distal half of small intestine; dotted column, cecum; and horizontally lined column, colon. Bars represent standard error of the mean.

function of diet; rats fed the stock diet had higher activity than either of the other groups. A similar trend was observed in the radiolabeled sterol recovered from the colon but no statistically significant difference was found. Decreasing amounts of labeled acidic steroids were found in the more distal segments of the intestine. Diet affected acidic radioactivity in the large intestine to a statistically significant extent; cecal radioactivity was significantly different among all three groups.

Radiolabeled steroids excreted in the feces were found in significantly different concentrations among the dietary groups when the data were expressed either in terms of concentration or total excretion (Table 2). The stock diet-fed rats excreted the largest weight of feces which were characterized by lower amounts of both neutral and acidic steroids. The differences in concentration of fecal radioactivity between the rats fed commercial ration and the other two groups, although still statistically significant, were not as great when total excreted radioactivity was calculated.

Determination of total recovered radioactivity from all samples assayed from the rats fed the stock diet was 42 $\pm$ 3% of the administered dose, which was significantly lower than the SP or SP plus cellulose groups which gave 54 $\pm$ 5% and 55 $\pm$ 3%, respectively.

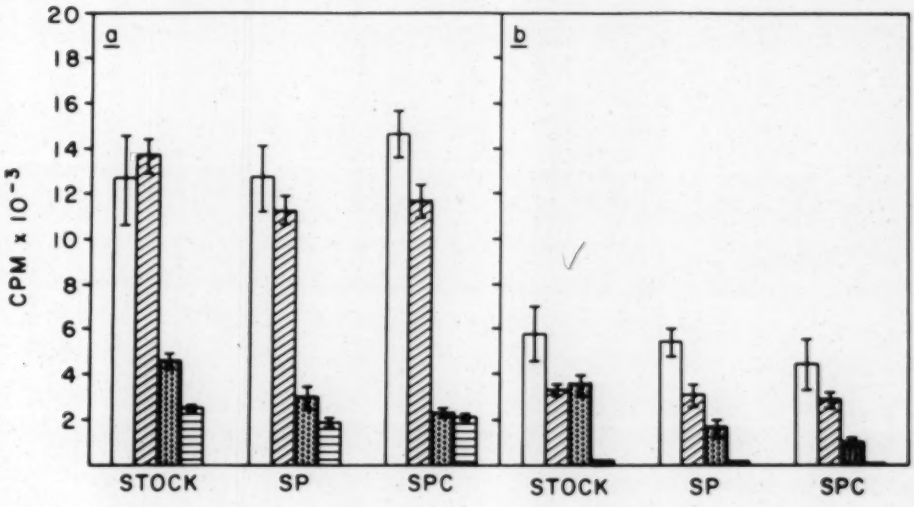

FIG 2

Radioactivity recovered from intestinal tissue associated with neutral steroids (2a) and acidic steroids (2b). Open columns, stomach and proximal half of small intestine; diagonally lined column, distal half of small intestine; dotted column, cecum; and horizontally lined column, colon. Bars represent standard error of the mean.

DISCUSSION

Our data show that significant amounts of exogenous cholesterol and its acidic derivatives remain in the intestines up to 3 days following administration of radiolabeled sterol. Diets characterized by differences in the non-digestible carbohydrate content significantly affected the concentration of both neutral and acidic steroids in the large intestine and feces but not the small intestine. The overall pattern of steroid distribution throughout the GI tract was not affected by diet. These findings are in general agreement with those of Peng et al. (10) who compared the mean relative specific activity of cholesterol in the mucosa and lumenal contents of the GI tract with the plasma 10 days after a single intracardiac injection of labeled cholesterol. Those authors observed that the GI specific activity equaled that of the plasma. The fact that the present results show significantly more radioactivity in the gut probably stems from the different route of labeled cholesterol administration and a shorter time period between isotope administration and killing.

Large amounts of radioactive cholesterol were found in the wall of the small intestine along with increased labeled neutral sterol in the contents of

the large intestine. The most probable explanation for these findings is incorporation of significant amounts of exogenous cholesterol into cell membranes of epithelium lining the small intestine with subsequent extrusion and excretion through the colon. This uptake of cholesterol into the mucosal cells is presumably for structural purposes. This mechanism was postulated by Sodhi et al. (11) who suggested that the cholesterol derived from rapidly exfoliated mucosal cells at the distal ends of villi is not absorbed readily. This hypothesis is supported by our findings on cholesterol distribution throughout the intestine. Recently, Miettinen et al. (12) presented evidence suggesting that a significant portion fecal cholesterol originated in the intestinal wall by secretion rather than from the plasma.

The relatively poor absorption of cholesterol in both humans (13) and rats (14) is well established. In the present experiment, between 7 and 9% of the administered dose of labeled cholesterol was recovered from the serum-liver pool. The amount retained in the intestines was equal to that absorbed. Considerable losses of labeled sterol were observed in all groups, with a significantly greater loss in the stock diet-fed group. This suggests that intestinal flora, which are modified by diet, also may play an important role in recovery of cholesterol via degradation of cholesterol to ketone bodies or other metabolites. Other parameters that play a role in recovery of sterol and may differ in rats fed a stock diet versus an SP diet are transit time or physical entrapment of sterol in a polysaccharide matrix or some other type of binding (15).

Radiolabeled bile acid distribution patterns were similar among all groups. Only the concentrations were significantly influenced by the type of dietary fiber. This is analogous to a previous study of bile salt distribution in the small intestine (5).

A mechanism likely to account for the differences in intestinal sterol concentrations as a function of diet is differential bile acid-binding by fiber. Kritchevsky and Story (16) found little bile acid-binding by cellulose. Components of stock diets, such as lignin, bound substantially more bile acids (17). Our data on acidic steroid radioactivity in the contents of the large intestine are compatible with the results from in vitro investigations. It is apparent that significant amounts of radiolabeled cholesterol remain in the GI tract for several days following oral administration, both as neutral sterol and as acidic metabolites.

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BIOAVAILABILITY OF VITAMIN E IN RATS FED DIETS CONTAINING PECTIN

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ABSTRACT

Weanling male Sprague-Dawley rats were fed:

(1) a basal diet without supplemental vitamin E (-E),
(2) a basal diet supplemented with 39 IU vitamin E/kg of diet (+E) or
(3) an E supplemented basal diet to which 10 percent citrus pectin was added (+E+P).

The animals were studied over a 56 day period. Growth rate as measured by body weight was noticeably slower in the +E+P group as compared to both the -E and the +E groups. The final average body weight of the +E+P group was 28 percent less ($P<0.05$) than the final average body weight of the +E group. No difference was observed in body weight between the +E and the -E groups. Plasma and red cell vitamin E levels in both the -E and the +E+P groups decreased rapidly after starting the dietary regimen. Parallel with this rapid decrease in red cell vitamin E level was a rapid increase in the in vitro peroxide hemolysis value in both the +E+P and the -E groups. At the end of the study, plasma vitamin E values (mg/dl) were 0.13±0.1(-E), 0.04±0.2 (+E+P), and 1.08±0.17(+E) and red cell vitamin E values (ug/ml) were 0.44±0.10(-E), 3.49±0.30(+E+P), and 5.74±0.37(+E). Vitamin E contents of other tissues including liver, heart and lung were 3 to 4 fold lower in both the +E+P and the -E group compared to the +E group. These results indicate that the consumption of a diet high in pectin content reduces the bioavailability of vitamin E.

KEY WORDS: Pectin, vitamin E, dietary fiber, bioavailability.

INTRODUCTION

Pectic substances or pectin, along with cellulose, hemicellulose, and lignin constitute some of what is now called dietary fiber. Pectin has been demonstrated to have important physiological and nutritional effects such as: reduction in food intake, weight, absorption of protein, lipid, and calcium (1,2); decrease in digestible energy of basal ration, reduced absorption of heavy metals and toxic materials (3); increased excretion of fecal sterols and lipids, binding of bile salts (4,5); and binding of polyvalent cations and increased requirement for vitamin B_{12} (6). Pectin had been shown consistently to decrease plasma and liver cholesterol levels in a number of experimental animals (4). This reduction was accompanied by increased bile acid excretion. These observations are consistent with a mechanism wherein pectin binds bile acids resulting in changes in cholesterol absorption and excretion. A consequence of bile acid binding is that it becomes unavailable for micelle formation which is necessary for absorption of biliary and dietary cholesterol, thus leading to lower cholesterol absorption. Lipids and lipid-soluble vitamins are also dependent upon bile acids for absorption; therefore, decreased availability of bile acids for micelle formation would be expected to have an adverse action on the absorption of other essential lipids. Two studies at low-levels of pectin ingestion have been reported with conflicting findings (7,8). Our studies were undertaken to determine the effects of high levels of dietary pectin on the bioavailability of vitamin E in rats.

MATERIALS AND METHODS

Diet and Feeding. Forty-two male weanling Sprague-Dawley (Simonsen Lab, Gilroy, CA) rats housed in individual steel cages were fed initially a diet containing 44 IU vitamin E/kg diet for 6 days. Then 6 rats were killed to provide zero day biochemical indices. The remaining rats were divided equally (12 rats/group) into 3 dietary groups: control, 10 percent pectin and vitamin E-deficient (-vitamin E) (Table 1). Both control and vitamin E-deficient groups were fed the same amount of feed consumed by the pectin group (paired fed). Another group with 12 rats was fed the control diet ad libitum to provide baseline data for differences in feed consumption. Weight gains were recorded every 4 days. Blood samples were collected weekly by tail tip slice and were analyzed immediately for percent red blood cell (RBC) hemolysis, plasma, and RBC vitamin E. After 28 days, 6 rats from each diet group were killed, and blood, liver, heart and lung tissues were collected for vitamin E analysis. After 56 days, the remaining rats were killed and the same tissues were collected for vitamin E analysis.

Pectin used was commercially available citrus pectin which contained 9.7 percent methoxyl group (49 percent esterified) according to our analysis using the method of McCreedy (9).

Table 1

Composition of Test Diets

	Conditioning Diet	Paired Control	10% Pectin	Minus Vit. E
	g/kg	g/kg	g/kg	g/kg
Stock based[1]	266	266	266	266
Sucrose	677.6	677.6	577.6	677.8
Fat (stripped corn oil)	50	50	50	50
Potassium acetate	6.27	6.27	6.27	6.27
Vit. E (D, L α-tocopherol acetate) 0.25 IU/mg	0.176	0.154	0.154	0
Pectin[2]	0	0	100	0

[1] Basal diet contains the following ingredients in g/kg: Casein, 200; mineral mix A, 40; mineral mix B, 4; vitamin mix 10, choline bitartrate mix 10; DL methionine 2, the vitamin mix is made up of the following in g/kg: thiamine-HCl, 0.125, riboflavin, 0.25, pyridoxine-HCl 0.7, niacin 1.5, Ca-α-pantothenate 0.8, vitamin B_{12} 1% trituration in mannitol, 0.5; biotin, 0.03; menadione, 0.05; folic acid, 0.2; vitamin A palmitate (250,000 IU/g), 0.8; vitamin D_2 (calciferol, 500,000 IU/g), 0.2.
choline bitartrate mix is made up of: choline bitartrate 67.5 g, sucrose 435.5g.
Mineral mix A is made up of the following in g/kg: $CaHPO_4$ 364.7; $CaCO_3$, 44.45; Na_2HPO_4, 77.25; KCl, 52.5; $MgSO_4$, 49.5
Mineral mix B is made up of the following in g/kg: $CuSO_4$, 3.1; ferric citrate 104.8; $MnSO_4$ H_2O 76.9: $ZnCO_3$ 11.5; KIO_3 0.06; Na_2SeO_3 0.05; NaF 0.55 and KCr $(SO_4)_2$ 12 H_2O 0.72). Sucrose to make 1 kg.

[2] Pectin (Sunkist Grower, Inc. Corona, CA) contained 9.7 percent methoxyl group according to our analysis.

Vitamin E Analysis. Heparinized whole blood samples were centrifuged at 2,000 rpm (500 x g) for 5 min to remove plasma and whole blood cells. Red blood cells were then washed twice with 5mM phosphate buffered saline, pH 7.4 and analyzed for vitamin E by the modified method of Taylor et al. (10). This method involves saponification in the presence of large amounts of ascorbic acid, extraction of the non-saponifiable lipids with hexane, and fluorimetric measurement of the tocopherol. The non-saponifiable lipid phase contains only one fluorochrome in the 290 nm excitation and 330 nm excitation range which had been identified as tocopherol (10). The usual saponification mixture contained 2.0 ml of 5mM phosphate buffered saline, pH 7.4, 0.5 ml of 25 percent ascorbic acid (prepared fresh for each determination), 1.0 ml of 100 percent ethanol, and 0.5 ml of washed RBC (ca. 50 percent in hematocrit). The mixtures were pre-incubated at 70°C for 5 min in glass stoppered 15 ml centrifuge tubes. Following addition of 1.0 ml of 10N KOH, the mixtures were saponified for 30 min at 70°C. After cooling, 4.0 ml of hexane (glass-distilled) was added and the saponified mixture was extracted by mixing for 1 min on a vortex mixer. The extracted mixtures were centrifuged in a clinical centrifuge for 5 min and a portion of the hexane phase was removed for fluorimetric measurement.

The heart, liver and lung samples were homogenized (1:4) with 5 mM phosphate buffered saline, pH 7.4 containing 10 percent ascorbic acid. The homogenate was centrifuged and the supernatant was analyzed for vitamin E as above.

Hemolysis Test. Red blood cells were washed 3 times with 25mM phosphate buffered saline (PBS), pH 7.4, and resuspended to 4 percent hematocrit with the same buffer. An equal volume of PBS containing 2 percent hydrogen peroxide was added to each RBC suspension. The reaction mixtures were incubated at 37°C for 3 hours. After incubation, the amount of hemolysis was determined by measuring the optical density of each supernatant at 540 nm. The percent hemolysis was expressed as the ratio of the amount of hemolysis in each sample containing H_2O_2 to that in 100 percent hemolysis. The 100 percent hemolysis sample was prepared by adding equal volume of distilled water containing 2 percent H_2O_2 to the 4 percent RBC suspension.

Statistical Analysis. Data were analyzed using one-way analysis of variance. When significant differences were observed, the significance of the differences among the means was determined by Tukey's Honestly Significant Test Difference (11).

RESULTS AND DISCUSSION

The group of rats fed pectin ate less food compared to the ad libitum control group of rats (Fig. 1). Since the purpose of including the ad libitum group was to demonstrate reduced food intake of the pectin-fed group, the ad libitum control group was terminated after 30 days. To eliminate the effects due to differences in food intake, the control and vitamin E-deficient groups were fed the same amount of food as the pectin group. Even with similar food intake, the average weight of the pectin rats was 25 percent less than either group ($P<0.05$). Assuming that the pectin, which replaced sucrose in the diet, did not contribute any metabolic energy to the diet, the pectin-fed rats would be getting only 10 percent less calories than the other respective groups. The decreased weight gain could in part be explained by the reduced absorption of lipids and other nutrients due to absorption to dietary pectin as shown by other studies (1,2).

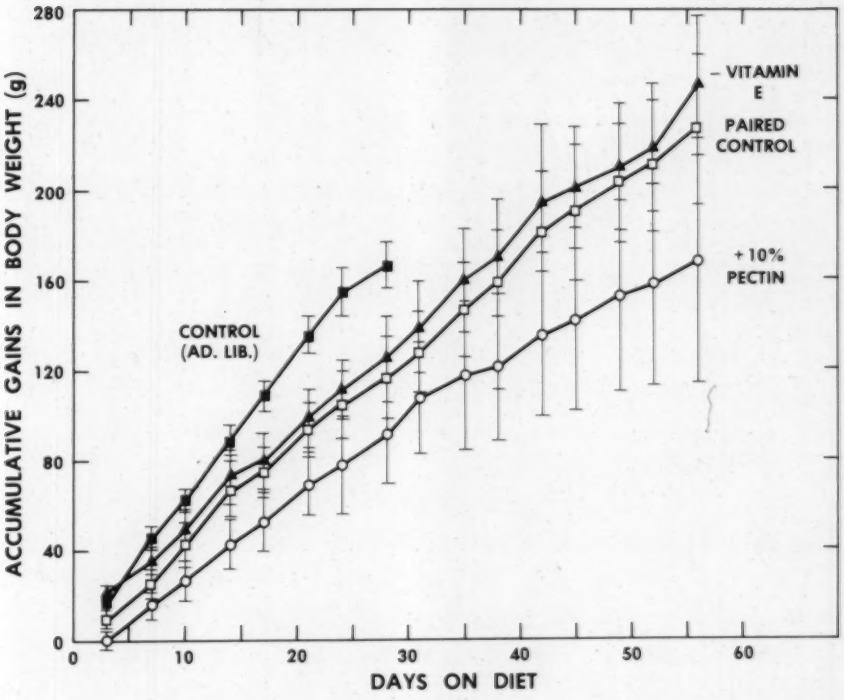

FIG. 1

Effect of diets on weight gains of rats. Each point represents the mean ± S.D. Paired control and -E groups were fed the same amount of diet as the pectin group.

Erythrocyte hemolysis is one of the more commonly used biological assays to assess vitamin E content in blood. Percent hemolysis increased significantly in both pectin and vitamin E-deficient groups after only one week of feeding (Fig. 2). Thereafter, the pectin group value decreased to an average of 71 percent while the vitamin E-deficient group remained at 98 percent and the paired-control was 4 percent. The mean values, averaged over the period 14 to 56 days, were statistically ($P<.05$) different from each other, that is, the percent hemolysis of the vitamin E-deficient group was higher than the pectin group which in turn was higher than the paired control. The rapid rise in the hemolysis rate during the first week of feeding in the vitamin E-deficient and pectin groups was paralleled by a decrease in vitamin E levels in RBC.

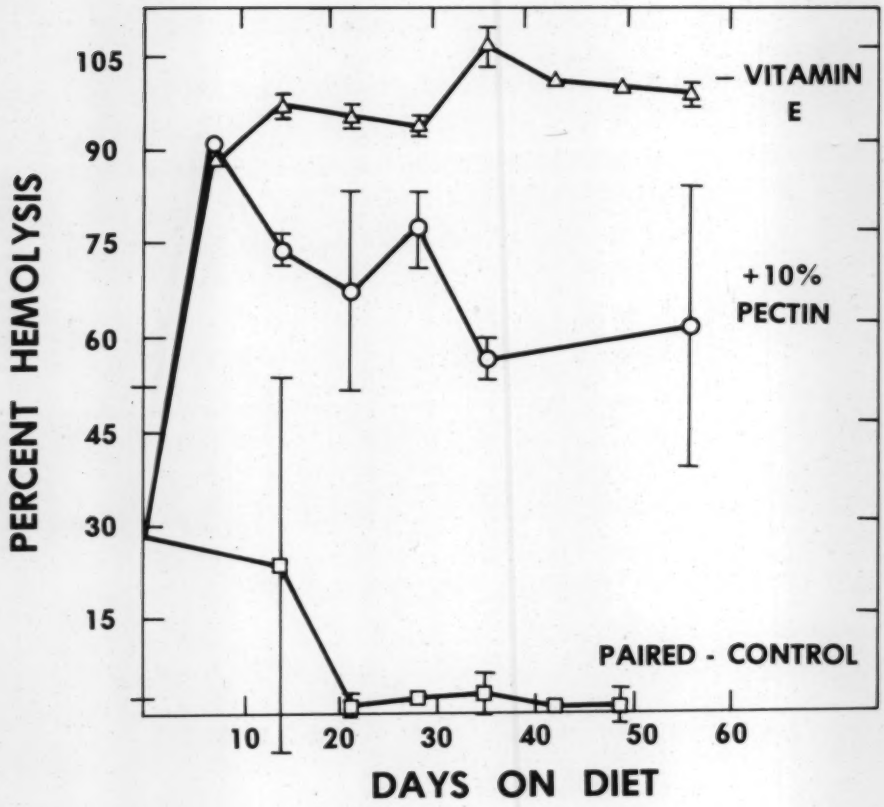

FIG. 2

Effect of different experimental diets on red blood cell hemolysis. Each point represents the mean±S.D.

Plasma and RBC vitamin E levels of the pectin group were consistently lower than the paired control but higher than the vitamin E-deficient rats (Fig. 3). The values for RBC vitamin E averaged from the 7th day through the 56th day were 0.48±0.31 mg/dl (-E), 3.28±0.79 mg/dl (+E+P), 5.02±0.61 (+E). All are significantly different ($P<.05$) from one another, supporting the in vitro hemolysis test data. The plasma tocopherol contents, determined through the same period were: 0.20±0.1 mg/dl (-E), 0.44±0.07 mg/dl (+E+P) and 1.06±0.28 mg/dl (+E). The value for the pectin group was significantly lower ($P<.05$) than the control, but not different from the vitamin E deficient rats. The drop in RBC tocopherol of the pectin group during the first two weeks of feeding was paralleled by a similar decrease in the vitamin E-deficient group. Thereafter, the values increased up to 35 days and leveled off. It is not known whether this pattern is significant; however, further study is warranted. The pattern is suggestive of an adaptation mechanism in which a rapid initial decrease in vitamin E levels is followed by an increase and levelling off. Bieri (12) demonstrated that for most tissues of rats, dietary depletion of vitamin E caused an initial, relatively rapid loss followed by a slower, prolonged withdrawal of the vitamin. No data was reported for RBC.

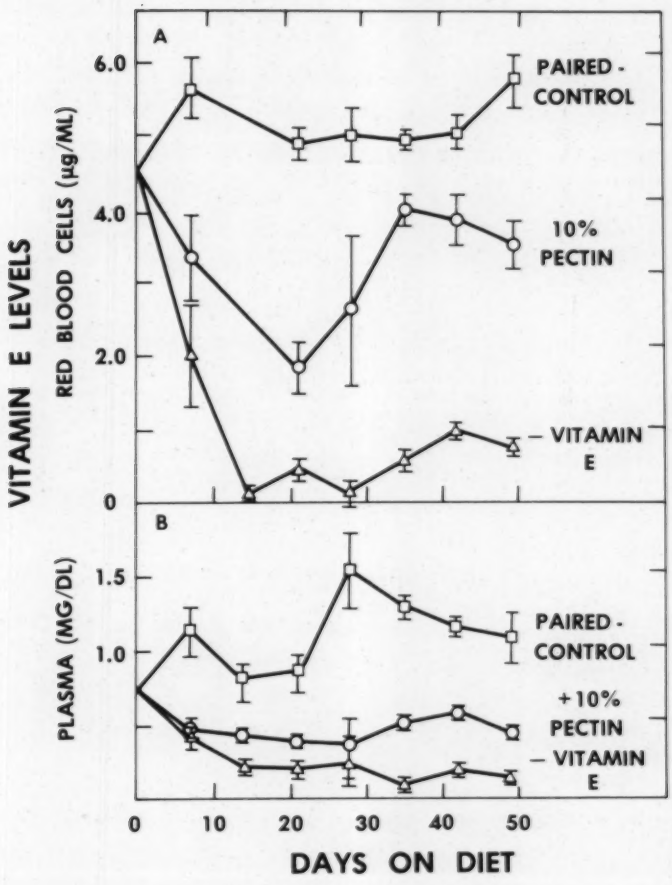

FIG. 3

A. Effect of different diets on red cell vitamin E levels of rats.

B. Effect of different diets on plasma vitamin E levels of rats. Each point represents the mean ± S.D.

On the 28th and 56th day of feeding, vitamin E contents of the liver, heart, and lung samples were 3- to 4-fold lower in the pectin and vitamin E-deficient groups compared to the paired control (Table 2). There was no statistically significant difference between pectin and vitamin E-deficient groups, suggesting that the effect of vitamin E deprivation was similar to feeding 10 percent pectin in the diet in depleting the tissue reserves. Since there were only two time points measured, it was impossible to determine whether or not the rates of depletion in the two groups were similar or not. Nonetheless, the data showed that vitamin E bioavailability to the tissues was reduced significantly by pectin in the diet.

The vitamin E contents of the tissues in the pectin and vitamin E-deficient animals on the 28th day of feeding were generally lower than on the 56th day. The differences were not statistically significant; but the trend possibly reflects the losses in vitamin E during frozen storage of the 28th-day samples which were not analyzed until the 56th day samples were available.

Plasma cholesterol levels decreased in the pectin group after 28 and 56 days of feeding (Table 3). The RBC cholesterol content was not affected. This confirms the studies that demonstrated lowering of plasma cholesterol as a result of feeding pectin to rats (4).

Table 2

Vitamin E Content of Various Tissues from Rats Fed Test Diets

Diet	Vitamin E Content (μg/gm tissue)					
	28 Days Feeding			56 Days Feeding		
	Liver	Heart	Lungs	Liver	Heart	Lungs
Paired-Control (+E)	8.26±1.37	9.26±2.74	8.39±1.65	8.15±1.25	10.02±2.37	8.86±3.24
10% Pectin (+E+P)	1.87±1.07	1.58±0.96	2.52±2.52	2.62±1.18	2.33±1.25	2.94±1.20
Minus Vitamin (-E)	0.88±0.74	0.95±1.17	1.31±1.67	2.48±1.79	2.15±1.80	2.67±1.26

Values are mean ± S.D. with N = 6.

All values of the pectin group and the minus vitamin E group are significantly different from the control group ($P < 0.01$).

Table 3

Plasma and Red Blood Cell Cholesterol of Rats Fed Test Diets

Diet	Cholesterol Content (mg/dl)			
	Plasma		Red Blood Cell	
	28 Days Feeding	56 Days Feeding	28 Days Feeding	56 Days Feeding
Paired Control (+E)	88.0±26.9	85.0±12.6	126.2±33.2	134.8± 4.4
10% Pectin (+E=P)	48.8±25.8	41.3± 7.2	120.0± 7.3	146.5±21.2
Minus Vitamin E (-E)	80.9±28.7	60.1± 4.4	121.5± 7.1	153.2± 7.7

Values are mean ± S.D. of N = 3.

Red blood cells cholesterol levels of the different diet groups are not significantly different from each other.

Plasma cholesterol levels of the pectin are significantly different from the control group at $P < .01$ while that of minus vitamin E are not.

Overall, all the indices measured showed that dietary pectin decreased the bioavailability of vitamin E. Further, the effects could be observed after a week of feeding. In experiments on rats, Phillips and Brien (7) found that pectin added at 3 percent of the diet did not affect the liver storage of vitamin A nor was there any evidence for limited utilization of the pro-vitamin β-carotene. Kasper et al. (8) demonstrated in human subjects that several dietary components, including pectin, when fed separately increased absorption of vitamin A as measured by serum levels. To our knowledge, our report is the first report showing adverse effects of pectin consumption on the absorption of vitamin E.

A point to consider is the high amount of pectin that was used in our experiment, which was 3 times higher than that used by Philipps and Brien (7) in their vitamin A experiments. There are, however, other studies that had used levels of pectin within the range we used. Rao and Bright-See (13) fed rations to rats containing 0-20 percent pectin and found that food intakes and weight gain decreased in rations containing 12 percent or more pectin. The amount of pectin used in human studies was from 12 to 50g/day (14,15). This is equivalent to 0.6 to 2.5g/100 kcal (assuming an intake of 2000 kcal/day) and would be comparable to that of Rao and Bright-See, i.e., 4, 8, and 12 percent pectin (0.87, 1.83 and 2.88g/100 kcal) and to our study, i.e., 10 percent pectin (2.5g/100 kcal). In our study, we deliberately exaggerated the diet with 10 pectin, to represent a high fiber composition. Lower levels of pectin will be used in future experiments to measure dose-response.

These results are consistent with the working hypothesis that pectin binds bile acids and makes them unavailable for micelle formation, and consequently decreasing the absorption of lipid-soluble components such as vitamin E. Because of the nonspecific nature of the interaction of the bile-lipid soluble components, it is possible that the absorption of other fat soluble vitamins and nutrients were also adversely affected at the level of pectin administered. The pectin-fed rats, in addition to being smaller, had non-glossy, dull coats, exhibited loss of hair on the ventral side and were weaker compared to the paired control. Thus, it is possible that there was reduced intake of vitamins A and D and essential fatty acids due to decreased lipid absorption and perhaps other nutrients. Furthermore, vitamin E has been demonstrated to interrelate with other nutrients such as vitamin A, iron ascorbic acid, thiamin, vitamin B_{12}, vitamin D_2, manganese and magnesium (16). Vitamin E has long been known to have a sparing effect on liver vitamin A (17). The low levels of vitamin E in the tissues of the pectin-fed rats, combined with the increased demand for antioxidants due to the 5% corn oil in the diet may have adversely affected the vitamin A status of the animals. The nutritional status of the rats with respect to nutrients other than vitamin E, was not evaluated in this study. Future studies should take this into consideration.

At this time it is difficult to extrapolate this preliminary information to humans. Considering an average feed consumption of 16g/day for a 160g rat, the rats in the pectin group consumed about 0.012 g pectin/day/g body weight. A dietary fiber consumption of 8.1 g/day/person in the U.S. (18) is calculated to be 1.2×10^{-4} g/day/g body weight for a 70 kg adult. If all the dietary fiber were pectin, the amount fed to the rats was several times that of human dietary fiber consumption in the U.S. when expressed in the units above. The consumption of a diet containing 10 percent pectin represents an artificial diet and may only simulate the upper limits of a total vegetarian diet. Clearly, any dietary recommendations for humans require further research.

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EFFECT OF ALTERED LITTER SIZE ON THE CELLULARITY OF SKELETAL MUSCLES AT WEANING AND SUBSEQUENT GROWTH IN RATS

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ABSTRACT

The growth of skeletal muscles in rats suckled in various sized litters was studied. Litters were divided at 2 days post partum into: small (3 pups), medium (7-9 pups) and large litters (15-20 pups). Males from all three litter sizes were killed at the end of lactation; another group of males from these litters was fed a stock diet ad libitum to 143 days of age. At both 23 and 143 days of age the rats suckled in large litters had lighter bodies and muscles than animals from small or medium sized litters; at 23 days of age the muscles had significantly smaller fibers but there was no reduction in the number of fibers. These differences in muscle cellularity did not exist at 143 days. Bone length was reduced also in the rats suckled in large litters at 23 and 143 days of age. Feeding the rats from the large litters until they attained the body weight of those suckled in small litters produced a return of the weight and fiber diameter of the muscles to those in the small litters. The length of the humerus, and therefore the biceps brachii, was longer, thereby giving a different conformation to the muscle. The extent of the differences in all measurements between the litters at 143 days of age was less than those at 23 days of age. The data indicate that the number of fibers in skeletal muscles is not affected by a nutritional stress imposed during lactation; thus catch-up growth occurs by hypertrophy.

Key Words: Lactation . Rat . Litter size . Skeletal muscle . Fiber number . Fiber diameter .

INTRODUCTION

Alteration in the number of pups suckled by rats fed an adequate diet has been used to study catch-up growth (1), adipose tissue cellularity (2), carcass fat (3), the growth of various tissues (4,5) and the composition of the milk (6). No data are available on the number and diameter of fibers of different skeletal muscles from rats at constant age and at constant body weight when suckled in small, medium and large-sized litters. The suckling period is a critical stage in the development of skeletal muscles in rats (7). One study has shown adverse effects on hypertrophy and hyperplasia in a skeletal muscle from rats suckled in large sized litters (4). Hypertrophy and hyperplasia of muscle fibers in that study were determined from DNA, RNA and protein concentrations. However, this approach has been questioned by recent studies on skeletal muscles during normal growth (8,9) and after acute dietary restriction (10) in the rat. The present study utilizes a technique that simultaneously determines the number and diameter of fibers in a complete cross-section of muscle. The study was undertaken because of the major contribution of skeletal muscle to total body mass. The influence of alteration of litter size on muscle cellularity at weaning and during the subsequent growth of skeletal muscles could thus be determined.

MATERIALS AND METHODS

Animals and diet

Pregnant Sprague-Dawley rats weighing about 260g, and bearing their first litter, were received from ARS, Madison, WI. The animals were housed separately in plastic cages containing sawdust bedding. Rat chow (Ralston Purina, St. Louis, MO) and tap water were available ad libitum to all animals used in this study. Room lighting was on a 12h. light and 12h. dark cycle. Room temperature was constant at 23°C.

Experimental design

The number of pups per litter was determined at 2 days postpartum. Adjustments were made to produce the following sized litters: small-sized (3 pups per litter), medium (7-9 pups per litter) and large-sized (15-20 pups per litter). Each category consisted of four litters. Litters born on the same day only were combined. Total weight of the litter was recorded at 23 days postpartum. Some of the pups from the large-sized litters were fed for an additional 5 days when they had achieved the body weight of the 23 day old pups suckled in small litters. This group is designated large-older. This experiment was repeated under identical conditions three months later. The two trials are referred to as Experiment 1a and 1b in Table 1. The difference in body weight of male rats from the small- and medium-sized litters varied between Experiment 1a and 1b (non-significant and $P < 0.005$, respectively. This inability to ensure similar body weight changes in response to identical stress situations was encountered previously (11). A review of the literature indicated wide variations in the body weight of rats from standardized litters ranging from small to large-sized (Table 2). This table is not a complete review of the literature, but it does illustrate the difficulty in drawing specific conclusions from data derived from this experimental design. When the body weight data from Experiment 1a and 1b in Table 1 were pooled there was no significant difference between the small and medium-sized litters at 23 days of age. The data presented in Table 3 were obtained using a sub-sample of randomly selected rats from Experiment 1a and 1b.

Further groups of male rats from the various sized litters in Experiment 1a were fed the stock diet until 143 days of age; n=14 in small-sized litters, n=7 in medium, n=14 in large-sized litters. The large-older group (n=14) took an average 40 days extra to reach the body weight of the 143 day old rats suckled in small-sized litters.

Postmortem procedures

Animals were killed by ether anesthesia and the carcasses were allowed to enter rigor mortis at 22°C. At 4h. postmortem the biceps brachii from the forelimb and the soleus from the hindlimb were dissected from origin to insertion according to the description of Greene (12). Random selection was made of the side from which the muscle was dissected. Muscles were weighed and placed in a 10% neutral formalin solution buffered with 0.IM potassium phosphate for a minimum of 3 weeks. The number of muscle fibers and their diameter were determined on complete cross-sections of the muscles by using the Coulter Counter (Coulter Electronics, Hialeah, Florida) as described by Thompson et al. (13).

The humerus from the forelimb and tibia from the hind limb were dissected free of tissue, and their length was measured by a calipers. This measurement was used as an indirect assessment of the length of the biceps brachii and soleus muscles, respectively.

Data were analyzed by the Student's " t-test (14). Comparisons were made between the medium and the small or large sized litters, and between the large-older and small sized litters in Tables 1 and 3.

RESULTS AND DISCUSSION

When the number of pups in a litter of rats is altered soon after birth there are frequently difficulties in the interpretation of body weight data when the pups are weaned. The variation in body weight between pups raised in small- and medium-sized litters in two trials conducted under identical conditions in the present study (Table 1) and the variation obtained by other researchers (Table 2) illustrates this point.

TABLE 1

Body weight of rat pups suckled in small-, medium-, and large-sized litters. (Mean ± SEM)

Age (days)	Litter size (no. of pups)	Body weight (g)	
		Experiment 1a	Experiment 1b
23	Small (3 pups)	56.1±2.0	74.7±1.7
		N.S.[1,2]	**
	Medium(7-9 pups)	58.8±1.5	62.9±1.6
		***	***
	Large (15-20 pups)	36.2±0.8	45.1±0.5
		***	***
	Large-older	56.8±0.4	76.8±1.6
		N.S.[3]	N.S.[3]
143	Small (3 pups)	419±10	
		N.S.	
	Medium (7-9 pups)	399±9	
	Large (15-20 pups)	373±4	
		***	
	Large-older	395±4	
		N.S.[3]	

[1] Statistical comparisons are made between the following groups: small vs. medium; medium vs. large; large vs. large-older and large-older vs. small.

[2] N.S.= Non-significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

[3] Comparison between 'large-older' animals and those suckled in the small litters. The 'large-older' group were 5 days older than the 23 day old groups and 40 days older than the 143 day old groups.

TABLE 2

Some studies reporting body weight of rat pups at weaning after litter size alteration during suckling.

Pups per litter	Reference cited[1]														Treatment mean
	1	2	3	4	5a	5b	6	7	8	9	10	11	12a	12b	
	Mean body weight at weaning (g)														
2							44								44.0
3													56	75	65.5
4		43	40				48	48		57					47.2
6	62				62	56					64	53			59.4
8				45			44						59	63	52.8
10					53	43									48.0
12	44						44	32	59		59	20			43.0
16		23		32	45	32	28								32.0
18		18							29			19	36	45	29.4
20								29							29.0
22										15					15.0

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We conclude from the data in Tables 1 and 2 that the difference in body weight of weanling rats that were suckled in small-or medium-sized litters is not significant. There is general agreement from the data of the present study and from other studies that a large number of pups in a litter leads to a reduction in the body weight of individual pups at weaning (Table 1 and 2). When the animals were maintained on the stock diet until 143 days of age there was no significant difference between the body weight of rats suckled in small- or medium-sized litters (Table 1). A difference did exist between the body weight of these two groups and the group suckled in large-sized litters.

The weight of the biceps brachii and soleus muscles in the 23-day old animals (Table 3) reflected the trends in body weight described above. When the rats suckled in large-sized litters were fed until they reached the body weight of the animals from the small-sized litters, the weight of the muscles was not significantly different from that of animals suckled in small-sized litters.

TABLE 3

Muscle weight, the diameter and number of muscle fibers, and the length of representative bones in rats suckled in small-, medium-, and large-sized litters.

Litter size	Muscle wt. (mg)	Fiber diameter (μm)	Fiber number	Bone length[1] (cm)
Biceps brachii				
Small (23 days) n=7	43.5±4.0	24.1±0.8	6720±352	1.54±0.04
	N.S.	*	N.S.	N.S.
Medium (23 days) n=9	46.3±1.9	28.1±1.0	7604±331	1.59±0.03
	***	***	N.S.	**
Large (23 days) n=10	31.4±1.4	22.0±0.3	7905±563	1.49±0.01
	***	***	N.S.	***
Large-older (28 days) n=9	45.7±2.4	24.1±0.4	7846±486	1.65±0.03
	[2]N.S.	N.S.	N.S.	*
Small (143 days) n=12	270±8	50.0±0.4	7110±282	2.89±0.02
	N.S.	N.S.	N.S.	N.S.
Medium (143 days) n=7	253±11	49.9±0.6	6584±301	2.88±0.02
	N.S.	N.S.	N.S.	*
Large (143 days) n=5	237±11	48.7±1.1	7357±352	2.81±0.01
	***	N.S.	N.S.	**
Large-older (183 days) n=5	292±9	48.3±0.6	7353±341	3.01±0.05
	[2]N.S.	*	N.S.	*
Soleus (continued over)				

Litter size	Muscle wt. (mg)	Fiber diameter (μm)	Fiber number	Bone length[1] (cm)
Soleus				
Small (23 days) n=10	25.4±2.2	28.2±0.8	3015±149	2.31±0.05
	N.S.	N.S.	N.S.	N.S.
Medium (23 days) n=8	24.5±0.5	28.7±0.9	2747±155	2.43±0.04
	***	***	*	***
Large (23 days) n=10	16.6±10.9	23.5±0.8	3167±126	2.10±0.06
	***	N.S.	N.S.	***
Large-older (28 days) n=8	24.4±1.3	26.0±1.2	3197±216	2.48±0.06
	[2]N.S.	N.S.	N.S.	N.S.
Small (143 days) n=6	134±5	54.0±0.6	2296±106	4.21±0.04
	N.S.	N.S.	N.S.	N.S.
Medium (143 days) n=4	140±16	56.8±2.0	2164±343	4.28±0.02
	N.S.	N.S.	N.S.	**
Large (143 days) n=4	124±7	54.0±1.2	2225±217	4.10±0.02
	N.S.	N.S.	N.S.	*
Large-older (183 days) n=4	132±8	53.0±2.5	2274±85	4.27±0.06
	[2]N.S.	N.S.	N.S.	N.S.

[1]Humerus in the case of the biceps brachii muscle, and the tibia in the case of the soleus muscle.

[2]This line represents the statistical significance in data from the large-older group and the group listed as small litter size.

Significant differences in the diameter of fibers in two muscles were obtained when animals raised in small- or medium-sized litters were compared with those raised in large-sized litters at 23 days of age (Table 3). The deficit in fiber diameter when animals were suckled in large-sized litters was removed when the animals were fed until they reached the body weight of the 23 day old rats from the small-sized litter. The number of fibers in the muscles was not affected by the nutritional stress during lactation. This could be interpreted as being contrary to conclusions in an experiment using a similar design in which nuclear number was used as a basis for estimating cellularity (4). This provides another example of a situation in which the number of muscle nuclei is altered but the number of fibers is not (8,9). The explanation may be found in the reduced length of muscle fibers as indicated by reduction in bone length of the rats suckled in large litters (Table 3).

Animals suckled in large litters and then fed to the body weight of animals suckled in small-sized litters had longer bones than did animals suckled in small-sized litters (Table 3). Values were significantly different when

this comparison was made for the humerus in animals at both 23 and 143 days of age. Similar comparisons on the tibia showed no significant difference, but the trend in the data was the same as that observed in the humerus. These differences in the length of the humerus mean that, although the weight of the biceps brachii was the same in animals suckled in small-sized litters and in large-sized litters fed to the same body weight (as those suckled in small litters), the conformation of the muscle must have been different. This is due to the difference in the length of the humerus to which the biceps brachii muscle is attached. We suggest that longer muscles will have a greater DNA content than shorter muscles of the same weight.

Rats from the various sized litters fed the stock diet ad libitum to 143 days of age showed the same trends in body weight as shown by the 23 day old animals (Table 1). Therefore, there was no catch-up growth in body weight and this is in agreement with other studies (1, 16). However, the 38% difference in body weight at weaning between the rats suckled in small- and large-sized litters ($P < 0.001$) had decreased to a 11% difference at 143 days of age ($P < 0.05$). The absolute difference in weight was 20-30 grams at day 23 and about 45 grams at day 143. The weight of the biceps brachii and the soleus and the number and diameter of the fibers in these muscles were not significantly different between the small- and medium-sized litters or between the medium- and the large-sized litters when the rats were 143 days old (Table 3). These observations differ from those of others who found no catch-up growth in skeletal muscles when rats suckled in 18 and 9-12 pups per litter were fed a stock diet ad libitum from weaning to 130 days of age. A partial explanation for this difference may be provided in the muscles chosen for study. The weight of the gastrocnemius muscle chosen by Winick and Noble (16) increases considerably as a percentage of body weight during lactation and up to 45 days of age, whereas the increase in the soleus is minimal and the biceps brachii maintains a constant percentage of total body weight (17). A nutritional insult during lactation, such as that resulting from an increase in the size of the litter, may have more long-term effects on the more rapidly growing gastrocnemius. Catch-up growth in human skeletal muscles is an important aspect in rehabilitation from malnutrition in infancy as shown by Cheek et al.(18). If there is a differential response by individual muscles to nutritional stresses during lactation, then the choice of muscle for biopsy samples from children is of importance. A further consideration in the type of study reported here is the sex of the experimental animal. Female rats have a larger capacity than male rats for catch-up growth and recovery to control body weight (19).

The results of the present study indicate that muscle fiber number decreases between 23 and 143 days of age in both the biceps brachii and soleus muscles from all treatments with the exception of the soleus from animals suckled in small litters (Table 3). This is in agreement with previous observations made on rats during normal growth (9).

It can be concluded that a reduction in the normal supply of milk to suckling rats reduces the weight of skeletal muscles due to a reduction in the length (based on the length of the corresponding bones) and the diameter of the constituent fibers. There is no reduction in the number of muscle fibers. This information should be contrasted with results from similar type stresses that caused reductions in the number of cells in adipose tissue (2).

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TASTE ACUITY AND AGING: A REVIEW

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ABSTRACT

Studies on taste acuity and aging have been summarized with a review of the normal physiology of the sense of taste. The number of taste buds decreases with advancing age. Sweet and salt taste sensitivity are usually reported to decline with age. Changes in bitter sensitivity have been correlated with smoking. Denture wearers may complain of a decrease in taste when dentures are worn. Oral hygiene and a decrease in the sense of smell with aging are related to taste acuity. These changes in taste acuity with aging have important implications for nutrition counseling of older individuals.

KEY WORDS: Aging, taste

A surge of interest in various nutritional characteristics of elderly Americans has become apparent in recent years. There are approximately 24.7 million individuals over sixty-five years old in the United States (1) with the number expected to double over the next fifty years. Nutrient requirements are believed to remain at adult levels, except the calorie requirement which is lowered due to a fall in the basal metabolic rate and reduction in physical activity (2). Hence, a higher quality diet is needed to provide adequate quantities of nutrients without excess calories.

Many factors affect food intake of the elderly. Some of these include socio-economic status, ability to shop, life-long food habits, life style, dental health, pathologic conditions and mental status. Frequently diminished taste acuity is cited as a contributing factor.

Alterations in taste response have been associated with many pathologic conditions including hypertension (3,4), obesity (5-7), diabetes (8,9), cancer (10), adrenal cortical insufficiency (11), long-term hemodialysis (12), and hypothyroidism (13). Relationships between impaired zinc status (14,15) and experimental vitamin A deficiency (16) and abnormal taste sensation have been reported. In addition, a decline in taste acuity has been suggested to occur in healthy elderly as a natural process of aging. Evidence has been reported which both supports and refutes this concept.

The purpose of this paper is to review the physiology of the sense of taste and to review studies on taste thresholds and sensitivity done with healthy elderly subjects.

PHYSIOLOGY OF TASTE

Four basic tastes associated with food are detectable by humans - sour, salty, sweet and bitter. Humans are also able to detect an electric or metallic taste and a soapy (alkaline) taste sensation.

Sour taste is determined by the hydrogen ion content of the substance (17). Salt taste is less well understood, but is mediated by anion-cation interactions (18). The mechanisms of sweet and bitter tastes are more complex. Substances which vary greatly in structure can evoke the same taste, while certain substances with closely similar structures may have quite different tastes. For example, optical isomers of some amino acids stimulate taste, with the L-isomer perceived as bitter and the D-isomer as sweet (17). Thus for many substances, the taste cannot be predicted from physical or chemical properties.

Food tastes are multimodal; most food tastes are determined by mixtures of two or more of the basic tastes. The sensation and intensity of flavor also depend on signals from thermoreceptors, pressure receptors and pain centers in the oral cavity and throat, as well as the odor of the food (19). Table 1 summarizes factors which influence taste.

TABLE 1

Factors Influencing Taste Sensitivity

Condition of the mucous layer of the tongue and papillae
Number of taste buds stimulated
-- area of tongue covered
-- number of taste buds present
Duration of stimulus
Temperature of mucosa
Temperature of stimulus
Texture of stimulus
Odor of stimulus
Quantity and concentration of stimulus
Status of the nervous system
Adaptation to prolonged exposure to a stimulus
Saliva flow rate
Composition of saliva
Smoking history
Zinc status

Taste buds, the organs of taste, are located throughout the oral cavity. The greatest number are found in microscopic folds in the skin of the tongue called papillae (18). There are four different structural types of papillae. The fungiform papillae are found over the entire surface of the tongue. Vallate papillae are larger and fewer in number; adults have a total of seven to twelve. Foliate papillae are in folds found at the edges of the back of the tongue. They are well developed in children and less prominent in adults. Tastes buds are distributed among these three papillae types. The fourth type of papillae, the filliform, are dispersed over the entire tongue, but do not contain taste buds (17). Figure 1 is a representation of the histologic appearance of the papillae with taste buds.

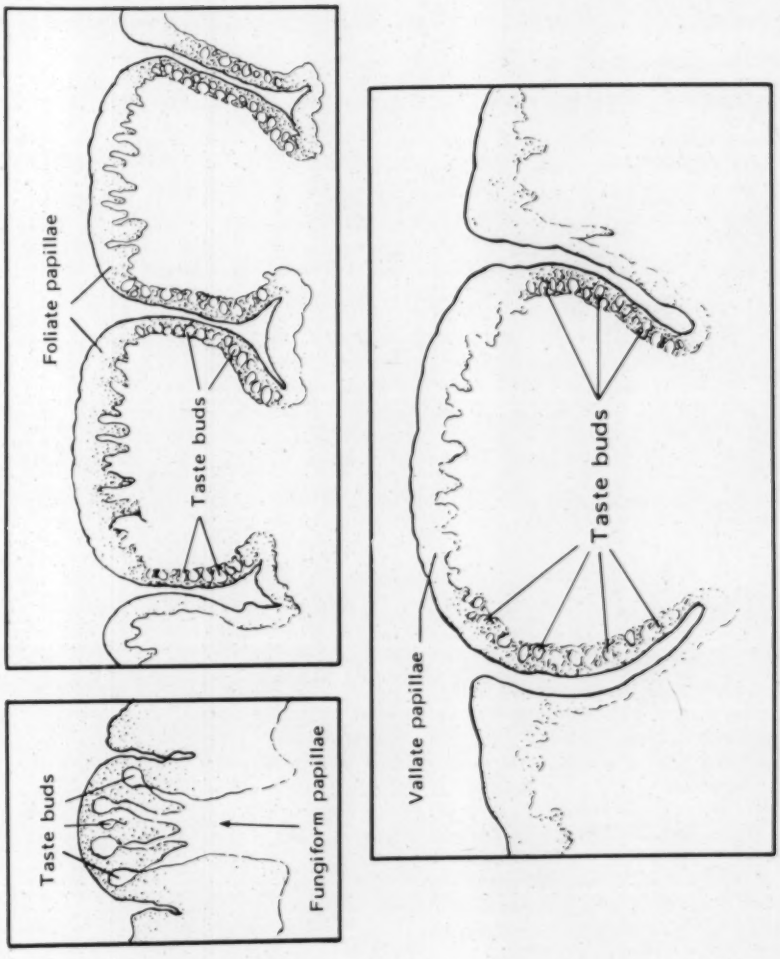

FIG. 1

Histological appearance of papillae with taste buds.

There are about 2,000 to 9,000 taste buds in the oral cavity (17,20) depending upon the investigator quoted. Each taste bud consists of forty to sixty spindle shaped cells which terminate in microvilli. The microvilli extend into the pores of the tongue and have direct contact with substances on the tongue.

A study in rats showed the life span of taste buds to be about ten days (21). Biedler reported (18) the same life span for human taste buds, with a range of three to thirty days. Normally, taste buds appear to degenerate and regenerate at the same rate. It has been suggested that with age, the rate of replacement of taste buds does not keep pace, leading to fewer taste buds (20).

Arey, et al. studied (22) the number of taste buds and papillae in human cadavers and found a large variation in the number of taste buds per papilla. In the group of subjects aged twenty to seventy years, there was a mean of 206 taste buds per papilla. A mean of eighty-eight taste buds per papillae was observed in the group aged seventy to eighty-five years. The investigators suggested that eighty-eight may be falsely high due to the inclusion of atrophied taste buds. They concluded that the loss of papillae and taste buds begins in middle age and continues with increasing age. Busse suggested (23) the loss begins at the tip of the tongue and progresses posteriorly.

Although taste receptors are located throughout the oral cavity, most attention is directed to the taste sensitivity of the tongue. Figure 2 is a schematic representation of the areas of the tongue with greatest sensitivity to the basic tastes. The back of the tongue is most sensitive to bitter. Sweet taste is detected at the tip of the tongue. The sides of the tongue are most responsive to sour and salty stimuli.

Innervation of the tongue is multiple and complex. The chorda tympani branch of the facial nerve extends to the anterior two-thirds of the tongue. The back of the tongue is innervated by the glossopharyngeal nerve. The vagus nerve is associated with the throat, pharynx and larynx. Damage to these nerves can result in loss of taste sensitivity. If the glossopharyngeal nerve is injured, the ability to taste bitter will be diminished. Damage to the facial nerve will result in loss of sensitivity to all tastes except bitter (17).

Several studies have demonstrated the existence of taste receptors on the hard and soft palates (24,25). Collings cited (24) histological evidence of taste buds on the soft palate. In his study with taste solutions, he found the soft palate was more sensitive to bitter than the tongue (24). Henkin and Christiansen studied (25) taste sensitivity in young adult volunteers by anesthesizing the palate and tongue on separate occasions and then administering taste solutions. They reported that all four basic taste modalities were separately detected by the tongue, palate and pharynx. Sensitivity for salt and sweet was greatest on the tongue. The palate was more sensitive to sour and bitter.

Nilsson reported (26) on taste sensitivity of the palate tested by electrogustometry. This technique uses an electrical stimulus to measure taste acuity. An electrode is placed on the area to be tested and the current is applied without the subject's knowledge, via a foot switch. Nilsson found (26) ninety-one per cent of young subjects (less than forty years) were able to detect the taste on the palate. The number of subjects with a detectable threshold declined with increasing age. The same investigator then tested the taste acuity of the palate with solutions of the basic tastes (27). The soft palate was only slightlty less sensitive than the tongue for the basic tastes.

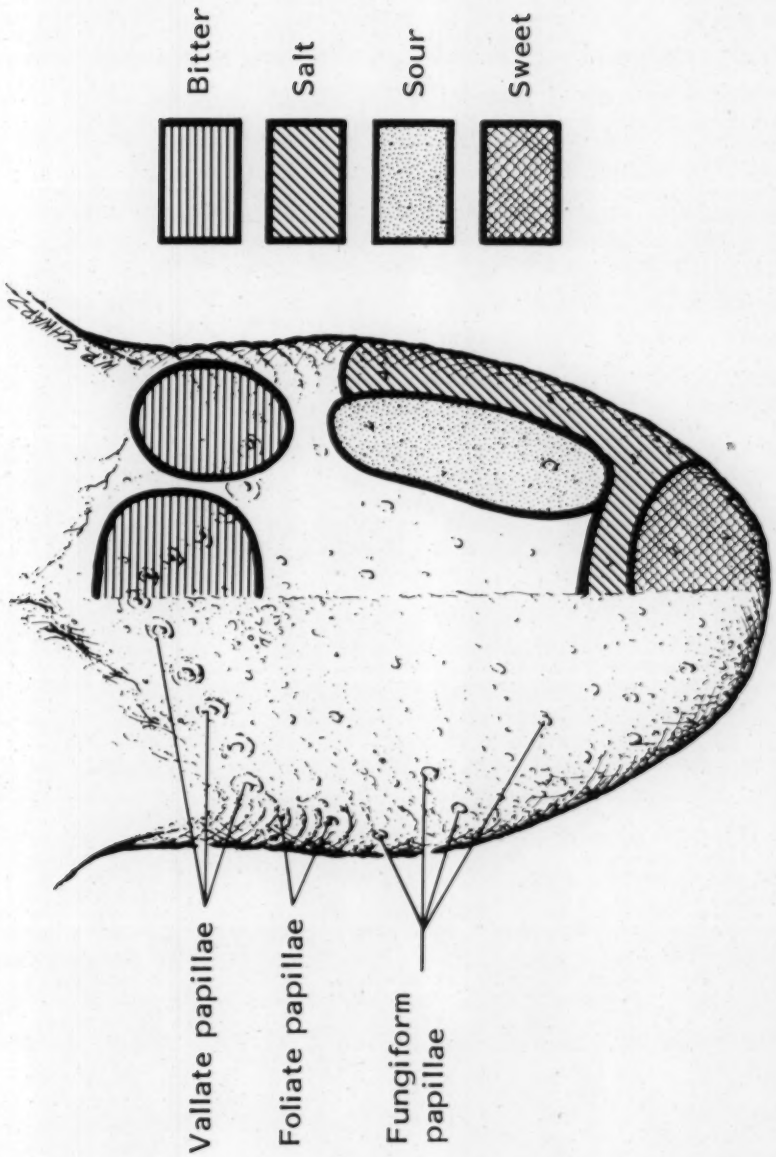

FIG. 2

Areas of greatest taste sensitivity on the tongue.

The hard palate was also sensitive, but to a lesser degree. The ability to recognize all four tastes was less frequent in the older subjects.

AGING AND TASTE ACUITY

The following terms are often used to describe taste acuity: Detection threshold is the lowest concentration of a taste solution which can be distinguished from water. Recognition threshold is the lowest concentration of a taste solution which can be correctly identified (11). Taste sensitivity is inversely related to taste thresholds. Thus, a high threshold indicates a low sensitivity to the stimulus.

Decreased taste sensitivity is often cited as a feature of old age. However, contradictory evidence exists in the literature regarding changes in taste acuity with age. Different methodologies employed for assessing taste acuity and the subjective nature of human response to taste stimuli need to be considered when attempting to draw conclusions from the available studies. Many variable factors, both nutritional and physiological, are associated with taste acuity (Table 1) which are difficult or impossible to control. One of these factors, zinc status, has recently been investigated in some elderly populations.

Sandstead suggested (28) some institutionalized individuals and some with low incomes have marginal to deficient intakes of zinc. Wagner, et. al. performed (29) hair and serum zinc analyses on low-income elderly subjects. They found thirty-nine per cent of those tested had low levels of one or both indices. Greger has investigated (30-32) zinc nutriture and taste acuity in noninstitutionalized and institutionalized elderly. She reported (30) the free-living subjects had a zinc intake equal to two-thirds of the Recommended Dietary Allowance. However, no correlation was found between dietary zinc intake and taste thresholds for sweet and salt tastes. In an institutionalized elderly population, no correlation between zinc intake, hair and serum zinc levels (31) or zinc supplementation (32) and taste acuity for sweet or salt stimuli was found. Hence, although the zinc status of the elderly may be marginal, no relationship between zinc status and decreased taste sensitivity has yet been demonstrated in this population.

The existence of fewer taste buds in older subjects (22) provides one explanation for the decline in taste acuity reported in some elderly individuals. However, a direct relationship between the number of taste buds present and the degree of taste sensitivity is not universally accepted (33). Other mechanisms for impaired taste acuity have been suggested. Nerve damage leading to paralysis or atrophy of the tongue mucous membrance have been cited as causes for total ageusia (34). Hinchcliffe suggested (35) that deterioration in brain cells may be responsible for diminished sensory response with age.

Some investigators have suggested that a decrease in taste acuity is not a normal process of senescence and occurs in relatively few of the elderly population. Shafar presented (36) four case histories of elderly patients with abnormal taste sensation. He felt the alteration in taste was due to a nonspecific hormonal or neuronal change and was not a normal event. Byrd and Gertman were unable to demonstrate a general decline in taste sensitivity with age, but did find a few elderly with marked impairment (37).

Cohen and Gitman surveyed (38) a group of noninstitutionalized persons. They found that thirty-three percent of the subjects over age sixty-five had general complaints regarding taste, compared to no complaints in the group younger than sixty-five. The most frequent problems were that food was sour,

bitter or dry. When taste perceptions were tested, no correlation between the subjective complaints and tested sensitivities were found. It may be concluded that factors other than taste contribute to food related complaints among the elderly.

Conversely, a general trend for impaired taste sensitivity with age has been reported (34,39). The decline seemed to become most apparent after the age of fifty years. All four taste modalities appeared to declince according to the same pattern (39). Degenerative changes in papillary structure and taste buds with old age were cited as the probable cause for a generalized decrease in taste sensitivity.

BASIC TASTES AND AGING

Table 2 summarizes studies on specific changes in sensitivity to the four basic tastes with aging.

SALT TASTE

There is agreement in the literature that sensitivity to salt declines to some degree with age. Hinchcliffe reported (40) a linear increase in salt threshold from age twenty to seventy with individual variations. He suggested the decline in sensitivity is a result of fewer taste buds with age. Balogh and Lelkes (34) and Cooper, et al. (39) also reported a decline in salt sensitivity. However, Hermel, et al. observed (41) no difference between young and old subjects.

An increased salivary sodium concentration has been observed in some elderly subjects (42). A higher salt taste threshold could occur due to adaptation to the taste of one's own saliva. The results of salt taste testing in this study showed elevated thresholds. The authors indicated the elevations were not large enough to imply the presence of a substantial deficit in salt taste acuity.

BITTER TASTE

Conflicting data exist regarding bitter taste sensitivity with advanced age. Glanville, et al. found a decline in bitter taste sensation with age (33). Males showed a greater deficit, with the sex difference increasing in magnitude with advancing age. The next year, the same group of investigators reported (43) a study where smoking history was controlled. They observed a highly significant decline in sensitivity to bitter with increasing age in smokers. No sex difference was noted. Aging between sixteen and fifty-five years was not associated with a significant deterioration in taste sensitivity unless combined with smoking. A similar decrease in bitter sensitivity with no change in the other taste modalities has been reported in younger subjects who smoked (44). Longer duration of smoking was associated with a higher threshold. A significant decrease in the threshold for bitter was observed in subjects who quit smoking (45). On the other hand, Cooper, et al. failed to show a significant difference in tasting ability with age in subjects who were smokers (39). An increase in sensitivity for bitter with age was reported by Balogh and Lelkes (34). The increase was greater in smokers than in non-smokers.

Phenyl thiocarbamide is a substance which tastes bitter to genetically predisposed individuals. Kalmus and Trotter performed (46) a direct test of sensitivity to phenyl thiocarbamide by testing taste thresholds and retesting the same subjects ten to fifteen years later. They reported a mean threshold deterioration of approximately three per cent per year.

TABLE 2

Summary of Studies on Changes in Sensitivity to the Basic Tastes with Advancing Age

Investigators	Year	No. of Subjects	Basic Tastes[a] salt	sweet	bitter	sour	Comment
Richter and Campbell (47)	1940	174	*	D	*	*	Thresholds three times higher than young subjects.
Hinchcliffe (40)	1958	200	D	D	*	*	No sex differences.
Cooper, et al. (39)	1959	100	D	D	D	D	Sharpest decline after age 50, sour declined after age 60. No sex or smoking differences.
Balogh and Lelkes (34)	1961	150	D	D	I	N	Marked increase in bitter for smokers.
Kalmus and Trotter (46)	1962	110	*	*	D	*	Tested phenyl thiocarbamide sensitivity in same subjects after 10-15 years.
Glanville, et al. (33)	1964	676	*	*	D	D	Greatest decline in males.
Kaplan, et al. (43)	1965	395	*	*	D	*	All smokers. Found no age or sex differences in non-smokers.
Hermel, et al. (41)	1970	118	N	D	D	D	No statistical analysis.
Grzegorczyk, et al. (42)	1979	76	D	*	*	*	Elevated salivary sodium concentration.

[a] D = Decreased I = Increased N = No difference * = Not Tested

SWEET TASTE

Sensitivity to sweet taste has been shown to diminish with age (34,35,39-41,47). Richter and Campbell reported (47) their elderly subjects had sweet thresholds three times as great as young subjects.

Degree of overweight was shown to be negatively correlated with hedonic (pleasantness) ratings for sweet solutions (6), but a difference in sweet thresholds between normal weight and overweight subjects could not be demonstrated (7). Enns, et al. reported (5) higher preferences for sweet taste by older males. Overweight individuals indicated less preference for sweet taste than normal weight individuals. Interpretation of these results is difficult because only five male and seven female subjects were evaluated.

SOUR TASTE

Few reports are available in the literature concerning sour taste acuity in the elderly. Cooper, et al. in their report of overall declining taste ability in the elderly, showed that sour taste remained constant until age sixty, compared to age fifty for the other tastes (39). Glanville and his group showed (33) a decrease in sensitivity to sour which was more pronounced in males. However, in a later study by the same group, the difference was attributed to smoking combined with age (43). In older subjects who did not smoke, there was no difference in sour threshold from younger subjects.

GENERAL TASTE CHANGES WITH AGING

The literature cited in the previous section consisted of studies done with solutions of the basic tastes. The following section describes other strategies which have been used to test taste acuity in the elderly. These studies are summarized in Table 3.

A weak galvanic current applied to the tip of the tongue was used by Hughes to test taste acuity in 117 subjects (48). Twenty-nine young subjects (age twenty-one to thirty) were tested to establish a "normal" threshold. Results showed eighteen per cent of the young group needed higher than the "normal" amperage for detection, while eighty-four per cent of the elderly group was in this category. Subdividing the results for the elderly group into decades showed a progressive elevation in threshold with increasing age. Cigarette smoking had no effect on taste thresholds measured by this method.

Schiffman investigated (49) the ability of elderly and young subjects to identify blended foods while blindfolded. She found obese young subjects were able to accurately identify sixty-nine per cent of the foods; normal weight young subjects identified forty-one per cent of the foods. The older subjects named twenty-four per cent of the foods correctly. It was noted, but not measured by the investigator, that the elderly subjects were less able to detect food odors. Thus, loss of accuracy seemed to be based on cues from both taste and smell.

It has been suggested that the sense of smell declines more rapidly than taste (20). Few studies are available, but Schiffman demonstrated (49,50) that the elderly have a considerable reduction in the ability to detect odors compared to young individuals. This impairment was shown to affect the ability to identify foods (49) and may affect the enjoyment of eating.

Schiffman, et al. tested (51) the ability of subjects to taste nineteen L-amino acids and four hydrochloric acid amino acid derivatives. Elderly

TABLE 3

Summary of Studies on General Changes in Taste Sensitivity with Advancing Age.

Investigators		Year	No. of Subjects	Results
Cohen and Gitman	(38)	1959	348	General subjective complaints of altered taste did not correlate with tested taste acuity.
Hughes	(48)	1969	117	Used galvanic current to test tongue. Sensitivity declined with each decade. Eighty-four per cent of subjects over sixty-two had an abnormally high threshold.
Langan and Yearick	(52)	1976	23	Improved sensitivity to sweet and salt tastes with better oral hygiene.
Greger	(30-32)	1977-78	44-65	No correlation between zinc intake, hair zinc, serum zinc or zinc supplementation and taste thresholds for sweet and salty.
Schiffman	(49)	1977	56	Decreased detection of food odors and recognition of blended foods when blindfolded.
Nilsson	(27)	1979	80	Older subjects less able to recognize taste solutions on the palate.
Schiffman, et al.	(51)	1979	*	Significant decrease in sensitivity to the taste of amino acids.

* Not given

subjects had significantly diminished sensitivity with an average threshold two and one half times higher than young subjects.

Oral hygiene may play a role in taste. Twenty-three institutionalized elderly with some natural teeth and poor oral hygiene were tested for taste acuity (52). Half of the group then received professional oral hygiene care for a period of five weeks. The control group was visited by a dental hygienist, but did not receive care. Retesting of taste sensitivity showed a significant increase in sensitivity to sweet and salt in the treated group with no change in sour or bitter thresholds. There was no change in the control group. Interestingly, both groups had a significant increase in food intake at the end of the study period as measured by three day food records. Therefore, improved intake could not be attributed to increased taste sensitivity. Poor oral hygiene is suggested to affect taste by (1) physical impairment caused by debris covering the taste buds, and (2) taste fatigue due to constant stimulation by decaying material in the mouth. Bacterial products such as lactic acid may also mask flavor detection.

As noted previously, documented taste aberrations have been reported in a variety of pathological conditions. Many elderly suffer from one or more chronic conditions. It is possible that altered taste sensitivity could occur secondary to disease and in combination with the taste changes often observed in healthy elderly. Related to this is the fact that many prescribed drugs can produce an altered sense of taste, lingering unpleasant taste or dry mouth (53).

DENTURES AND TASTE ACUITY

Data from the Missouri Nutrition Survey showed (54) that in subjects older than fifty-nine years, fifty-six per cent of males and sixty-two per cent of females were edentulous. Forty-eight per cent of male subjects and thirty per cent of female subjects had dentures. One survey of denture wearers showed twenty per cent believed the dentures impaired the taste of food (55). Taylor and Doku found eighty per cent of subjects of both sexes complained of diminished taste immediately following fitting of dentures (56). The loss of taste was permanent in twenty per cent of the males and almost fifty per cent of the females.

Controlled studies of taste sensitivity with and without dentures have produced contradictory results. No difference was observed in patients with natural teeth compared to denture wearers in the study by Cohen and Gitman (38). Slight improvements in taste acuity while wearing dentures compared to the same subjects without dentures have been reported (57,58). These investigators suggested there are no taste receptors on the palate which would be covered by a denture plate. Conversely, Henkin and Christiansen found a significant decrease in sensitivity for sour and bitter tastes with dentures in place (59). The same researchers previously demonstrated the existence of receptors for sour and bitter tastes on the palate of young subjects (25). Hermel, etI al. also reported (41) improved taste sensitivity with dentures removed.

SUMMARY

Studies relating changes in taste sensitivity and aging have been reviewed. Contradictory data exists as to whether a decline in taste acuity is a normal process of aging or if it is a pathological condition occurring in relatively few individuals. Zinc status may play a role in taste acuity, but a correlation between marginal zinc status and depressed taste sensation has not been demonstrated in elderly individuals. It is accepted that the number

of taste buds and papillae declines with age. However, the significance of this is not adequately understood. Different methods of testing taste sensitivity and the results were described. A decline in sweet and salt taste sensitivity with age are most often reported. Sensitivity to bitter has been related to smoking. Dentures, sense of smell and oral hygiene may also contribute to tasting ability in older persons.

Taste should be considered as one factor which influences the food choices of the elderly. Changes in taste acuity with aging have important implications for those who give nutritional advice to older adults and for those who plan menus for nursing homes and nutrition programs for the elderly (60). Many women over fifty-nine years old may be underweight (54). Food intake may be encouraged in these individuals by serving appropriately seasoned meals. In view of the reported decline in salt taste acuity, salt restriction may not be the primary consideration in an underweight older individual with slightly elevated blood pressure. Excessive consumption of sweets by some elderly may be related to depressed sensitivity for sweet taste. During individual counseling, specific attention should be given to taste alterations reported by the individual. Informing the older adult and his family that taste loss often occurs with advancing age may alleviate concern. Encouraging the use of spices and flavorings in food preparation may enhance food acceptance and desire to eat in the elderly individual.

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SUMMARIES OF CONFERENCES

Fourth Canadian Ross Conference on Pediatric Research

Ross Conferences bring together a small group of individuals to discuss a topical subject in an informal and relaxed atmosphere. The 4th Canadian Ross Conference on Pediatric Research had as its theme Childhood Antecedents of Adult Disease and was held in the picturesque Laurentian mountains of Quebec. A number of topics were mulled over, many had a nutritional theme. JOHN UDALL discussed the absorption of macromolecules in the gastrointestinal tract and how early feeding practices can alter this step. JOHN GERRARD related his extensive experience in the field of food intolerance and cited several case histories to support the concept that hypersensitivity to food antigens may underlie much common and bizarre syndromes. ALEXANDER FERGUSON described the effects of intrauterine growth retardation on immunocompetence and concluded that fetal malnutrition both in man and animals results in marked depression of cell-mediated immunity. Impaired immunocompetence in small-for-gestation low birth weight infants may persist for many months and perhaps years. WILLIAM DIETZ outlined the implications of childhood obesity and the factors which allow its persistence into later life. Excess dietary intake is one of the conglomerulate of factors contributing to the chronic vascular complications leading to premature mortality. PAUL PENCHARZ described the results his study on weight reduction among obese children and adolescents. DOUGLAS HOLSCLAW discussed the implications of cystic fibrosis for adults. As many as 50% of such patients are now surviving well into the third decade and the internist who looks after them has to be fully aware of the many physical, emotional and educational needs of the patient. Cystic fibrosis rarely may remain undiagnosed until adulthood. CHARLES SCRIVER highlighted the importance of genetic endowment as a critical determinant of disease and cited examples of phenylketonuria and hyperlipoproteinemia to illustrate the role of dietary environmental factors in the phenotypic expression of these inherited disorders. JUNE LLOYD described the London experience of managing children with hypercholesterolemia and the longterm prognosis even after rigid dietary control. ROBERT KRAMER briefly outlined the problem of alcohol abuse in North America and the environmental cluster which promotes such abuse. BARTON SCHMITT discussed the problem of child abuse in which deliberate nutritional deprivation is common, particularly among preschool children. Failure of thriving which cannot be readily explained on the basis of a physical disorder should alert the health professional to the possibility of child abuse and a team approach is required to reach a definite diagnosis and offer lasting solution.

The conference was held November 22-25, 1981 and was organized and chaired by RANJIT CHANDRA. Edited transcripts of the meeting would be available shortly through Ross Laboratories, Montreal.

Trace Element Regulation of Immunity and Infection

The Clinical Nutrition and Early Development Branch of the National Institute of Child Health and Development supported a workshop on the interactions of trace elements in immunity and infection. The meeting was organized and cochaired by RANJIT K. CHANDRA and DELBERT H. DAYTON and held in Bethesda September 16-18, 1981. An extended summary of the deliberations would be published shortly.

In the introductory session, QUENTIN MYRVIK outlined the host defenses which prevent the entry of pathogens into the body and how the immune system deals with those microorganisms which break through this barrier of skin and mucous membranes. VINAY KUMAR elaborated on the role of natural resistance, particularly natural killer cells. HAROLD SANDSTEAD provided the nutritional aspects of trace element physiology and function and underlined the important interactions among elements. GERALD KEUSCH briefly emphasized the nutrition-immunity relationships, particularly from the point of view of the invading pathogen. The role of zinc in modulation of immune responses and susceptibility to infection was summarized by RANJIT CHANDRA. It was emphasized that zinc deficiency, both primary and secondary, impairs cell-mediated immunity, particularly T helper cell function and exerts a selective effect on vulnerability to challenge with different microorganisms. GABRIEL FERNANDEZ and PAMELA FRAKER elaborated on this theme and presented their own original data. GERALD COOMBS summarized the limited data on selenium effects on immune response and highlighted the important interaction with vitamin E. JOSEPH PROHASKA described the influence of copper deficiency on cell-mediated immunity and antibidy response. The frequency of iron deficiency worldwide, the diagnostic criteria for confirming depletion of iron stores and the pathogenesis of impaired immunity associated with iron deficiency were presented by PETER DALLMAN. GEORGE HASS described the interesting finding of lymphoreticular hyperplasia seen in some animals with magnesium deficiency and considered the possibility of latent virus activation. Other presentations focussed on less well studied areas, including iodine (ROBERT CLARK), minor trace elements (FORREST NIELSON, MILOS CHVAPIL, JANET GREGER) and heavy metals (WILLIAM BEISEL, MATTIE RAE FOX). The extensive discussion between the participants who included a mix of immunologists, nutrionists and active investigators in the subject brought out the discrepencies in the findings of various workers and outlined the areas which need attention in the future.

ANNOUNCEMENTS

Nutritional Support of Specific Disorders

The City of Hope National Medical Center will hold a symposium on this topic. Among the subjects to be covered are nutritional assessment (Donald McLaren), nutritional management in gastro-intestinal disease (Lyn Howard), renal disease (Joel Kopple), psyhological aspects (Alfred Coodley), cancer (Michael Mequid), immunity and infection (Ranjit Chandra), brain function (Richard Wurtman, trauma (George Sheldon). Workshops and case management sessions are planned. The symposium will take place March 6-7,1982 at the Huntington-Sheraton Hotel, Pasadena, California. For more information, write Dr. Michael Meguid, City of Hope National Medical Center, 1500 East Duarte Road, Duarte, CA 91010.

Nutrition and Coronary Heart Disease

The German Foundation of Heart Disease Research will hold a conference on this topic at the Sheraton Hotel, Munich, March 15-16, 1982. The conference themes include the influence of diet composition from the experimental viewpoint on the genesis of arteriosclerosis, influence of fat intake on vascular disease from the epidemiological point of view, influence of nutrient proportion on human metabolism. For further information, write Kongressburo, Deutsche Herzhilfe e.V. Clemensstrasse 86, D-8000 Munchen 40, West Germany.

AIN/ASCN/CSNS Joint Meeting

The triennial joint meeting of the American Institute of Nutrition, American Society for Clinical Nutrition and Canadian Society for Nutritional Sciences will be held at the Pennsylvania State University, July 22-24, 1982. For information, write the society office, American Institute of Nutrition, 9650 Rockville Pike, Bethesda, Maryland 20814.

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EDITORIAL

Provision of nutrient supplements to pregnant women

In these times of budget constraints, it is likely that the number of large-scale field studies in nutrition intervention will be fewer than during the past 20 years. Such studies are costly, and difficult to design, control and evaluate. Nevertheless, they have provided invaluable information on the factors which interact with an inadequate food supply to result in suboptimal nutritional status, and modify the effectiveness of various forms of nutrition intervention.

Several of these studies have focussed on pregnant women, a group especially vulnerable to nutrient deficiencies, and one in which the effects of supplementation are relatively easy to measure. The project carried out between 1969 and 1977 by the Institute of Nutrition of Central America and Panama (INCAP) in Guatemala is a classic example of a large-scale intervention project in undernourished pregnant women. Provision of calories, vitamins and minerals improved many pregnancy outcome measures, including maternal weight gain, placental weight, birth weight, survival, infant growth, and neonatal neuro-motor adequacy and interaction (1-4). Improvement in another outcome measure can now be added to this list; in this issue of Nutrition Research, the Guatemalan group reports for the first time that energy supplementation may have increased the duration of gestation.

Five large-scale trials of prenatal supplementation have been made during the last decade (5). In Guatemala (1), New York (5), and Taiwan (6), the supplements were given to individual women as liquid formula diets; in Colombia (7), milk, oil and enriched bread were supplied to the entire family, and in Montreal (8), the women were provided with nutrition education, and foods to take home. These studies lead to a measurable improvement in several parameters of pregnancy outcome. Protein supplements produced no additional increase in birthweight above that due to their calorie content (1,5,6); they may even have caused a higher incidence of premature births and infant deaths among relatively protein-sufficient women in New York (5), although the validity of this conclusion has been questioned (9).

The challenge is whether, or not, we can use the experimental data on hand to make appropriate decisions about the optimum nutrient content of prenatal supplements, and the best method of delivering them to pregnant women. A comparison of high calorie, protein-containing supplements to low calorie supplements has been made in three studies where vitamins and minerals were also provided (1,5,6). However, no systematic, large-scale evaluation of the relative effectiveness of different prenatal nutrients or nutrient combinations has been attempted.

An additional problem is the form in which nutrients should be provided. If energy is indeed the most limiting nutrient, is it reasonable to supply it in the form of refined carbohydrates and separated fats? Can we be certain that the intake of micronutrients will be sufficient if such a policy is adopted? Providing prepared supplements to individual women is expensive, and if inappropriately formulated, increases the risk of dietary imbalances. It might also result in less subject compliance as compared to the distribution of real foods. However, if supplementary nutrients are supplied as commodities, it is unreasonable to expect that a substantial amount of these foods will not be consumed by other members of the family, or exchanged for goods or services.

Obviously, further research is needed to compare the effectiveness of different nutritional approaches in various local situations. The problem is how to answer the basic nutritional questions which still remain, while providing immediate practical and beneficial assistance to this critically vulnerable group.

Lindsay H. Allen

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NUTRITION AND LENGTH OF GESTATION

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ABSTRACT

This paper examines whether a food supplementation program carried out in Guatemala affected the length of gestation (n = 830 singleton births). Women who consumed more of a protein-energy or an energy supplement had significantly longer lengths of gestation. Regression slopes of energy on length of gestation were statistically significant and similar for both types of supplements, suggesting a role for energy but not for protein. The earlier calories were provided in pregnancy the greater their effect on gestation length. Women in the upper tercile of the distribution of energy intakes in the first trimester of pregnancy had a mean length of gestation 1.42 weeks longer than women in the lower tercile of the distribution. The percent of preterm deliveries (<37 weeks) was 5 percent in the upper group and 20 percent in the lower group.
KEY WORDS: gestation length, maternal nutrition, pregnancy, protein-energy malnutrition

INTRODUCTION

There are epidemiologic data which suggest that maternal nutritional status can affect the length of gestation. For instance, Vaughan (1) reported that three times as many women who had gone into premature labor had diets which were considered poor when compared with those of a control population. Low pregravid weight, inadequate weight gain during pregnancy, and poor socio-economic status have been identified as significant risk factors of a preterm delivery (2-4). Poor hematological status has also been shown to be associated with greater than expected preterm rates (5). Few reports of the results of nutrition experiments are available. Mora et al. (6) reported no effect of food supplementation during the last trimester of pregnancy on length of gestation and Rush et al. (7) observed an increased incidence of premature deliveries in women receiving a high protein supplement during pregnancy.

The present study examines whether a food supplementation program carried out in Guatemala affected the length of gestation. Previous publications have described in detail the population, experimental design, and methodology of the Guatemalan study and have reported that caloric supplementation during pregnancy improved birth weight (8), the weight of the placenta (9), and infant growth (10). This is the first publication from the Guatemala study to focus on gestation length.

MATERIALS AND METHODS

The data presented here are drawn from a longitudinal study of nutrition, physical growth, and mental development which the Institute of Nutrition of Central America and Panama (INCAP) carried out between January 1969 and

February 1977 in four small Spanish-speaking Guatemalan villages. The study population and the methods used have been described in detail elsewhare (8-10).

In 1969, INCAP began a preventive and curative medical care program and a food supplementation program. Health care was provided by auxiliary nurses under the supervision of a physician. Two types of liquid supplements were used. In two of the villages, a high protein-calorie supplement called "atole" containing 11.5 g of protein and 163 kcal per 180 ml was made available. The other two villages were provided with a fruit-flavored drink called "fresco" which contained no protein and supplied only 59 kcal per 180 ml. The supplements also contained vitamins and minerals (8). Attendance at the food supplementation centers and consumption of the supplement were free and voluntary, and as a result, a wide range of supplement intake during pregnancy was observed. The supplement was given twice daily, seven days a week. Subjects were provided with a cup containing 180 ml, and more was given if requested. Leftovers were measured and actual intake on an individual basis was recorded to the nearest 10 ml. Because dietary intake during pregnancy was more limiting in energy than in protein and because previous analyses have shown energy and not protein intake was related to pregnancy outcomes such as birth weight (8) and weight of placenta (9), supplement intake was expressed in terms of calories.

Pregnancies were identified as early as possible. At the end of the first trimester of pregnancy women were asked to recall the date of onset of the last menstrual period. From these data, length of gestation was calculated as the interval in days between the date of onset of the last menstrual period and the date of delivery.

Home dietary intakes were measured once at the end of each trimester of pregnancy using the 24-hour recall technique. Anthropometric examinations were also carried out with the same periodicity. Information about maternal morbidity during pregnancy was obtained from symptom-oriented household surveys carried out every two weeks. Other data available and used in this report were maternal age at delivery, parity, birth interval, and number of months women breast fed during pregnancy. Finally, a family socioeconomic scale was generated from information about home construction materials and number of rooms.

After delivery, children were examined periodically and data on nutrition, health, and development were collected until seven years of age. Birth weight was obtained within 24 hours after delivery by personnel using carefully standardized procedures and calibrated scales.

Between January 1, 1969 and February 28, 1977, there were 830 singleton deliveries for which length of gestation and food supplementation data were available. Four hundred and forty-one cases were from the atole villages and 389 were from the fresco villages. Birth-weight data were available for 721 of these 830 births. Mortality data were not available for 62 cases of the 830 births.

RESULTS

Means and standard deviations for some maternal characteristics during pregnancy are shown in Table 1 for atole and fresco villages. Home diet energy and protein intakes appear to be similar in atole and fresco villages. Energy intake from the supplements increased from the first to the third trimester of pregnancy and was greater in atole than in fresco villages. For all of pregnancy, average intake of calories from the supplements was significantly greater ($p < .001$) in atole villages (107 kcal/day) than in fresco villages (81 kcal/day).

Table 1

Mean and Standard Deviations for Some Maternal Characteristics During Pregnancy

	Atole villages		Fresco villages		Pooled standard deviation
	n	$\bar{X}$	n	$\bar{X}$	
Home diet[+]					
Energy (kcal/day)	341	1473	275	1411	467
Protein (g/day)	341	42.3	275	42.4	13.4
Supplement (kcal/day)[++]					
First trimester	441	69	389	39	81[c]
Second trimester	441	114	389	95	102[b]
Third trimester	441	138	389	108	108[c]
Maternal and newborn characteristics					
Maternal height (cm)	437	149.0	381	149.0	5.2
Maternal head circumference (cm)	438	51.2	381	50.8	1.3[c]
Age (years)	440	28.2	389	27.9	7.3
Parity	441	4.1	389	3.5	3.2[b]
Length of gestation (weeks)	441	39.6	389	39.1	2.9[a]
Birth interval with prior delivery (months)	375	27.5	323	30.7	29.0
Birth weight (g)	387	3077	334	3027	461
Month of breastfeeding during pregnancy	346	1.7	288	1.5	1.9

[+]Average of surveys carried out at the end of the second and third trimesters of pregnancy.

[++]Each 100 kcal from the atole are accompanied by 6.1 g of protein. The fresco contained calories but not protein.

[a]$p < .05$; [b]$p < .01$; [c]$p < .001$.

The atole contributed 7.5 g of protein per day during pregnancy; the fresco did not contain protein.

Means and standard deviations for length of gestation and for a number of obstetric characteristics are also shown in Table 1. The length of gestation was longer in atole (39.6 weeks) than in fresco (39.1 weeks) villages ($p < .05$). When the sample was broken down by sex, the tendency toward greater values in atole villages was evident for male newborns (39.6 weeks and 38.8 weeks for atole and fresco males respectively, $p < .05$) but not for female newborns (39.7 weeks and 39.5 weeks for atole and fresco females respectively, N.S.). Mothers from atole villages also had larger head circumferences ($p < .001$) and greater parities ($p < .01$) as shown in Table 1.

Correlations between length of gestation and key independent variables are shown in Table 2. Energy intake from the supplements, whether accompanied by protein as in atole villages or not as in fresco villages, was significantly associated with length of gestation during the first and second, but not the third trimesters of pregnancy. Significant associations with length of gestation were also found with average intake during the entire pregnancy (i.e., all three trimesters). Other variables associated with length of gestation in circumference, birth weight, maternal age, and number of months combining pregnancy and lactation.

Slopes of regressions of supplement intake on length of gestation are shown in Table 3 for atole, fresco, and the combined sample. There were trends, none statistically significant ($p > .05$), for first trimester slopes to be larger than second trimester slopes and for values for fresco to be larger than those for atole. Regressions of third trimester intakes on length of gestation were not statistically significant ($p > .05$). In the combined sample, 10,000 kcal during the first trimester were associated with an increase in length of gestation of 4.2 days ($p < .0001$) and a similar amount during the second trimester was associated with an increase of 3.0 days ($p < .001$). Supplement intake during the first trimester of pregnancy was highly correlated with supplement intake during the second trimester of pregnancy ($r = .632$, $n = 830$, $p < .001$). Hence the slope of second trimester supplement intake on length of gestation was reduced from 3.0 ($p < .001$) to 1.1 days/10,000 kcal (N.S.) after taking into account intake in the first trimester. Each of the slopes shown in Table 3 was not appreciably changed after adjusting for the variables, other than supplement intake, which are listed in Table 3 (including those listed in the footnote to Table 3).

The range of supplement intake was arbitrarily divided into terciles for two variables: first trimester supplement intake and average intake during the entire pregnancy. The mean supplement intake as well as the mean duration of gestation for each of the terciles is shown in Table 4. The difference in mean length of gestation between third and first terciles was 1.42 weeks for first trimester supplement intake and 1.06 weeks for average intake during pregnancy. Analysis of variance showed that the mean duration of gestation varied significantly by category for both supplement variables. Similarly, the percent of newborns with short gestational age (<37 weeks) decreased signifantly from low to high categories of supplement intake as shown in Table 5. The findings in Table 5 were statistically significant in all but one comparison (i.e., for the variable "entire pregnancy" in fresco villages).

A total of 721 cases had information about length of gestation and birth weight. The percent of newborns with low birth weight (<2.5 kg) and short gestational age (<37 weeks) by tercile of supplement intake in the first trimester of pregnancy is shown in Table 6. Higher supplement intakes were clearly associated with lower rates of low birth weight and prematurity.

TABLE 2

Correlations Between Length of Gestation and Independent Variables[+]

Variable	Atole villages n	Atole villages r	Fresco villages n	Fresco villages r	All villages n	All villages r
Supplement energy						
First trimester	441	.127[b]	389	.180[b]	830	.152[c]
Second trimester	441	.120[a]	389	.143[b]	830	.136[c]
Entire pregnancy	441	.103[a]	389	.139[b]	830	.127[c]
Maternal head circumference	438	.117[a]	381	.062	819	.102[b]
Birth weight	387	.179[c]	334	.068	721	.129[c]
Maternal age	440	-.086	389	-.054	829	-.069[a]
Months of combined pregnancy and lactation	346	.015	288	-.153[b]	634	-.056

[+]Only variables with at least one significant association are included. Home diet calories, home diet protein, third trimester supplement intake, maternal weight (first trimester), maternal height, maternal weight for height (first trimester), parity, birth interval with prior delivery, maternal illness during pregnancy, and family socioeconomic status were not statistically associated with the length of gestation.

[a]$p < .05$; [b]$p < .01$; [c]$p < .001$.

TABLE 3

Slopes (days per 10,000 kcal) of Regressions of Supplement Intake on Length of Gestation

	First trimester calories	Second trimester calories	Third trimester calories	Entire pregnancy
Atole villages (n = 441)	2.8[b]	2.3[a]	0.6	0.8[a]
Fresco villages (n = 389)	7.5[c]	3.6[b]	1.7	1.5[b]
All villages (n = 830)	4.2[c]	3.0[c]	1.2	1.1[c]

[a]$p < .05$; [b]$p < .01$; [c]$p < .001$.

TABLE 4

Mean Length of Gestation (weeks) by Terciles of Supplement Intake During the First Trimester of Pregnancy and During All of Pregnancy+

Tercile	Sample size	Mean supplement intake (kcal/day)	Mean length of gestation (weeks)
First trimester			
Low	271	0	38.69
Middle	275	22	39.30
High	284	138	40.11
Entire pregnancy			
Low	275	13	38.84
Middle	275	78	39.39
High	280	191	39.90

+The pooled standard deviation of length of gestation was 2.88 weeks. The mean length of gestation varied significantly by tercile of first trimester supplement intake (analysis of variance: $F = 17.62$, $p < .001$) as well as by tercile of total supplement intake during pregnancy (analysis of variance: $F = 9.61$, $p < .001$).

TABLE 5

Percent of Newborns with Short Gestational Age (<37 weeks) by Terciles of Supplement Intake in the First Trimester of Pregnancy and in the Entire Pregnancy

Tercile	Atole villages (n = 441)		Fresco villages (n = 389)		All villages (n = 830)	
	n	%	n	%	n	%
First trimester+						
Low	27/140	19.3	26/131	19.8	53/271	19.6
Middle	7/124	5.6	26/151	17.2	33/275	12.0
High	7/177	4.0	7/107	6.5	14/284	4.9
	$p < .001$		$p < .05$		$p < .001$	
Entire pregnancy						
Low	21/130	16.2	26/145	17.9	47/275	17.1
Middle	10/138	7.2	23/137	16.8	33/275	12.0
High	10/173	6.0	10/107	9.3	20/280	7.1
	$p < .01$		$p > .05$		$p < .01$	

+Terciles as defined in Table 4. Test of significance refer to χ^2, df = 2.

TABLE 6

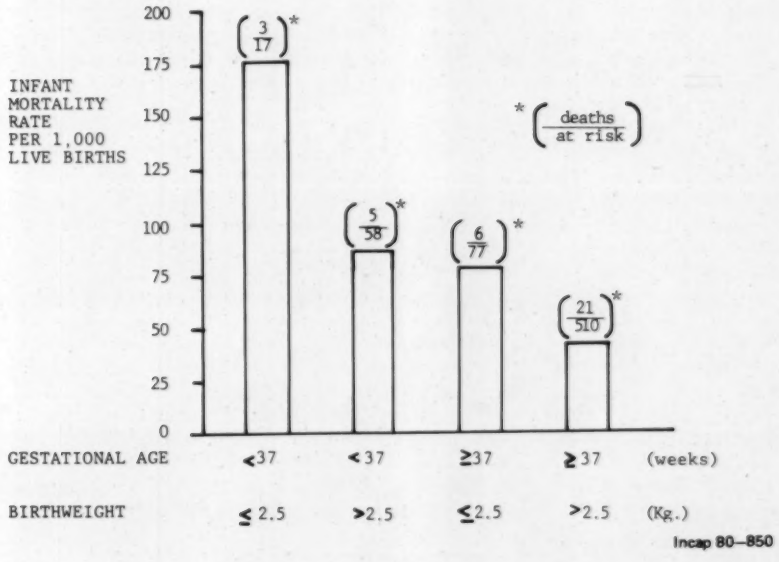
Percent of Newborns with Low Birth Weight (≤2.5 kg) and Short Gestational Age (<37 weeks) by Terciles (Low, Middle, High) of Supplement Intake During the First Trimester of Pregnancy (n = 721)

	Low		Middle		High		
	n	%	n	%	n	%	p
Low birth weight (≤2.5 kg)	38/203	18.7	25/252	9.9	34/266	12.8	<.05
Short gestation age (<37 weeks)	37/203	18.2	28/252	11.1	11/266	4.1	<.001
Low birth weight and/or short gestational age	66/203	32.5	29/252	19.4	40/266	15.0	<.001

+There were 721 cases with information about birth weight and length of gestation.

FIGURE 1

INFANT MORTALITY RATE BY CATEGORIES OF GESTATIONAL AGE AND BIRTHWEIGHT



Infant mortality data were not available for 59 of the 721 cases with both birth weight and length of gestation data. The remaining 662 cases were thus the data base for the analysis shown in Figure 1. Thirty-five of the 662 newborns died before one year of age. Those with low birth weight and short gestational age had the highest mortality (176/1000) while those with normal birth weight and gestational length had the least (41/1000).

DISCUSSION

The data presented show that the length of gestation increased as pregnant women consumed more of a protein-energy (atole) or an energy (fresco) supplement. As in earlier studies dealing with birth weight (8), weight of the placenta (9), and infant development (10), the hypothesis that the effects were due to calories rather than protein seems more plausible.

Whether the association between supplement intake and length of gestation is spurious or true is difficult to assess. The study design is quasi experimental in that villages rather than individuals were randomly assigned to treatment groups. The energy supplement was designed to be a placebo and the fact that fresco calories appear to be related to outcome variables in much the same manner as atole calories has left the study without a control group. Another problem is that the study was not preceded by a preintervention phase of data collection which would allow for "before-after" comparisons of groups.

The study did include data collection on a number of potentially confounding variables and the findings reported here indicate that the relationship between supplement intake and length of gestation remained statistically significant after adjusting for these variables. The best substitute for baseline data is the information collected during the first year of the project (i.e., 1969). The mean length of gestation in 1969 was 39.3 weeks in the atole ($n = 38$) and 39.2 weeks in the fresco villages ($n = 40$). Slightly higher values were observed for 1975-76, the last two years of the project (atole = 39.9 weeks, $n = 76$; fresco = 39.6 weeks, $n = 68$) but these were not statistically different from baseline values (pooled S.D. = 2.9, $p > .05$ for all comparisons). Though suggestive of improvement, the percent of cases with short gestational age (<37 weeks) did not change significantly ($p > .05$) from 1969-70 to 1975-76 in atole (15.8 to 6.6 percent) or fresco villages (15.0 to 10.3 percent. These analyses showing a lack of significant changes through time are biased against showing a nutritional effect in that all pregnancies occurring during the first year of the project were at least partially exposed to the supplementation program and hence not a "true" baseline.

Supplement intake was significantly associated with length of gestation but other variables indicative of maternal nutritional status were not. Home dietary energy and protein intakes as well as maternal stature and weight, were, for example, not associated with length of gestation. One explanation for the negative findings in the case of dietary variables is poor reliability and inadequate sampling of the mother's usual intake. Two interesting variables, maternal head circumference and months of combined pregnancy and lactation, did show significant associations with length of gestation. Because growth in head circumference is largely completed during the first few years of life, the data could be indicative of early nutritional status of the mother. Breast feeding during pregnancy certainly adds to the nutritional demands on the mother and would be expected to reflect negatively on pregnancy outcomes.

The mean length of gestation in the study villages ($\bar{X} = 39.4$, S.D. = 2.9. $n = 830$) was a week shorter than that reported for a California sample

($\bar{X}$ = 40.4, S.D. = 2.7, n = 198,408) (11). Also, the percent of preterm births (<37 weeks) was 11.9 in the study communities but 5.4 in the California sample (11) and 6.7 in the U.S. in 1967 (12). These differences cannot be said to be true differences because methodological nuances could account for the results. What is clear is that length of gestation, which was measured uniformly in the villages, varied with supplement intake. Women who were in the upper tercile of the distribution of supplement intake in the first trimester of pregnancy had a mean length of gestation 1.42 weeks longer than women in the lower tercile of the distribution. The percent of preterm infants was 5 percent in the upper and 20 percent in the lower tercile. Differences of these magnitudes, if true, would be of major biological significance in terms of birth weight, infant mortality, and development (13-16). The data shown in Figure 1 argue convincingly that effects of the above magnitude would be important in terms of infant mortality. Infant mortality rates were 133 per thousand for preterm infants but 46 per thousand for full term infants.

Supplement intake during the first trimester of pregnancy was more strongly related to length of gestation than intake during the rest of the pregnancy. In fact, supplement intake during the last third of the prenatal period showed no relationship at all with length of gestation. Interestingly, Mora et al. (6) improved the diets of women during the last three months of pregnancy and their results did not show modifications in the length of gestation.

In summary, the data from the Guatemalan study presented here are unique in showing consistent and biologically important associations between energy supplementation during pregnancy and length of gestation. Some alternative hypotheses were discarded by showing that the associations were independent of a number of potentially confounding variables. However, deficiencies inherent in the study design leave open many possibilities.

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ENERGY COST OF ACTIVITIES AND TASKS OF WOMEN FROM A RURAL REGION OF GUATEMALA

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ABSTRACT

The energy costs of various domestic and agricultural activities were measured by indirect calorimetry in 56 women, 16-49 years old, from a small rural village. Mean energy expenditures (kcal/kg/min) were 0.021 - 0.035 for fourteen activities ("light occupations"), 0.038 - 0.064 for thirteen ("moderately active" occupations) and 0.072 - 0.089 for four ("very active" occupations). There were no differences per kg of weight between pregnant, lactating and non-pregnant, non-lactating women. The results were compared with other studies and weighted averages were calculated. The values reported can be used in combination with measurements of time to estimate energy expenditure in time-motion studies.

KEY WORDS: energy expenditure; rural population; task performance; time and motion studies; women.

INTRODUCTION

There is little information on the energy requirements of women in rural, developing countries. Estimates have been based mainly on the requirements of urban women in industrialized regions, after taking into account the larger energy expenditure usually observed in rural women (1). The accuracy of such estimates must be established by gathering more information directly from rural women. The measurements of energy expenditure are of utmost importance to achieve this, since they are a major determinant of a person's dietary energy needs. One way of making those measurements is by time-motion studies (2,3). These entail two components: a) determination of the energy cost (i.e., the energy expended per unit of time) of the activities or tasks performed by a person, and b) measurements or estimations of the time devoted to the performance of such activities. Sometimes the measurement of energy costs is impractical or beyond the technical facilities of the investigator, or only the time estimates are available. Under those circumstances the values reported by other researchers for the energy cost of similar activities can be used. However, most of the information currently available on energy costs is from studies in men or in urban women. Few investigators have measured the energy expended during the usual activities of peasant women in rural regions (4-7), and measurements were usually performed only in small numbers of subjects. Furthermore, some of the activities measured have been defined in too general terms without considering that certain characteristics may influence energy demands.

This study provides actual estimates of the energy costs of the house-hold and agricultural activities most frequently performed by women in a rural region of Guatemala, describes how each activity was measured and compares the data with the results of other studies on women.

METHODS

Location: The study was made in Cacahuito, Taxisco, a village of 1043 inhabitants located 113 kms southeast of Guatemala City, at 616 m above sea level. It is in an area devoted mainly to the growing of coffee, corn, black beans and rice. Its population is an ethnic mixture of Spaniards and Maya indians with western cultural patterns ("ladinos"). Energy expenditure was measured in August-September, January-March and June-July. Environmental temperature ranged from 21 to 33°C, relative humidity from 41 to 96% and barometric pressure from 718 to 730 mm Hg.

Women: Fifty-eight healthy women between 16 and 49 years (mean ± S.D.: 27 ± 6), who accounted for about 30% of that age group in the village, participated in the study. Twelve were in the second or third trimester of pregnancy and 30 were lactating. Average weight was 49.1 ± 8.0 kg (range: 36.2 - 75.6) and average height was 150 ± 6 cm. They engaged mainly in child care and household-related activities and in chores such as gleaning, winnowing or chopping wood with a machete. Many women picked coffee during the harvest season.

Energy expenditure: Energy expenditure was measured by open-circuit indirect calorimetry, using a respiratory face mask with a low-resistance, small Rudolph valve. A portable respirometer of the Kofranyi-Michaelis type was used initially (8). It was substituted by a modification of the Douglas bag method (9), because most of the chores performed by the women produced low ventilation rates and required long periods to collect enough exhaled air in the rubber bag of the portable respirometer for an accurate analysis of its oxygen content. Briefly, it consisted of using a 3-way aluminum stopcock which connected a hose from the respiratory valve outlet to a 30-100 liter latex rubber balloon after 2 minutes for light tasks and 3-5 minutes for more strenuous tasks, all exhaled air was collected for 2-5 minutes; immediately afterwards the exhaled air was mixed by shaking the balloon, oxygen concentration was measured using a portable microfuel cell oxygen analyzer (Model 320-B, Teledyne Instruments, San Gabriel, Ca.) calibrated with certified gas mixtures, and volume was measured using a dry gas meter (Model 802, Singer, American Meter Division) or a Wright respirometer (Model P.M., British Oxygen Company) calibrated with a Tissot gas meter. The two methods and the similarity of the results obtained with them are described elsewhere (10). All women were accustomed to using the respiratory face mask and all other equipment prior to the actual measurements. Energy expenditure was calculated from oxygen consumption at standardized temperature and pressure (STPD), using 4.92 kcal/liter as the energy equivalent of oxygen.

Activities measured: The activities accounting for most of the women's day-time occupation were investigated. They are listed in Tables 1 and 2 and described in the Appendix. Most activities were measured in at least 4 women. The energy cost of the occupations described in Table 2 were estimated from direct observations of their activity components and calculations of the energy cost of those components.

RESULTS AND DISCUSSION

Table 1 shows the energy cost of the activities per unit of body weight and the corresponding average expenditures for the FAO/WHO "reference 55-kg woman" (1). Based on the classification of occupations suggested by the FAO/WHO Committee of Experts on Protein and Energy Requirements (1), activities 3 to 14 correspond to "light" occupations, activities 15 to 27 were "moderately active" and activities 28 to 32 were "very active". As might

TABLE 1

Energy Cost of Tasks Performed by Guatemalan Rural Women

Activity or task[a]	n[b]	Energy cost, kcal/kg/min Mean ± S. D.	Range	CV, %	Energy expended by 55-kg woman[c] kcal/min
1. Lying down	23	.021 ± .003	.017 - .026	13	1.14
2. Standing	8	.021 ± .005	.015 - .029	23	1.15
3. Buying or selling	d	.021			1.15
4-5. Sitting or sewing	20	.022 ± .044	.014 - .029	19	1.20
6. Nursing	d	.022			1.20
7. Eating	d	.022			1.20
8. Ironing clothes	1	.026			1.44
*9. Picking coffee	6	.027 ± .004	.023 - .033	14	1.50
*10. Winnowing or dekernelizing corn	15	.030 ± .006	.014 - .037	19	1.63
11. Washing dishes	1	.030			1.68
12. Cooking	19	.032 ± .008	.018 - .049	27	1.75
13. Personal necessities	d	.035			1.94
14. Hanging clothes	d	.035			1.94
15. Making tortillas	48	.038 ± .009	.017 - .056	23	2.08
16. Housecleaning	16	.040 ± .016	.020 - .077	40	2.20
17. Child care	4	.040 ± .010	.033 - .055	26	2.22
*18. Picking fruits	d	.044			2.42
*19. Weeding	d	.047			2.58
20. Washing clothes	16	.049 ± .010	.036 - .067	21	2.69
21. Walking on a flat terrain or downhill, with or without a load[e]	31	.050 ± .012	.032 - .078	24	2.73 (3.22)[f]
*22. Tending animals	d	.050			2.73
*23. Raking and burning	d	.050			2.77
24. Sweeping	33	.057 ± .010	.038 - .077	18	3.12
*25. Staking a fence	d	.058			3.18
*26. Cutting fruit with a pole	1	.061			3.34
*27. Light work with machete	d	.064			3.52
*28. Gleaning	5	.072 ± .026	.039 - .106	36	3.95
*29. Lifting and moving objects	4	.074 ± .018	.049 - .087	25	4.04
30. Walking uphill	18	.077 ± .019	.054 - .117	24	4.25
*31. Chopping wood with machete	6	.078 ± .013	.057 - .093	16	4.32
32. Carrying a load uphill[e]	24	.089 ± .016	.058 - .134	18	4.88 (5.77)[f]

[a] The tasks are described in detail in the Appendix. [b] n= number of observations; CV. = coefficient of variation. [c] WHO/FAO "reference woman" (1). [d] Estimated as described in Table 2. [e] Weight of the load must be added to the woman's weight to calculate energy expenditure. [f] In parentheses: energy expended while carrying a 10-kg load. *Tasks related mainly to agricultural activities.

TABLE 2

Energy Cost of Tasks Estimated from their Component Activities

Task[a]	Energy cost kcal/kg/min	Estimated as
Buying or selling	.021	- similar to standing
Nursing	.022	- similar to sitting or sewing
Eating	.022	- similar to sitting or sewing
Personal necessities	.035	- mean between standing and walking
Hanging clothes	.035	- mean between standing and walking
Picking fruits	.044	- mean of picking coffee and cutting fruit with a pole
Weeding	.047	- mean between sitting and gleaning
Tending animals	.050	- similar to walking
Raking and burning	.050	- mean of standing, sweeping and lifting
Staking a fence	.058	- mean of sitting or crouching, light work with machete and lifting
Light work with machete	.064	- mean between walking and chopping wood with machete

[a]The tasks are described in detail in the Appendix.

TABLE 3

Energy Cost of Activities Performed by Lactating, Pregnant and Non-Pregnant, Non-Lactating Women (NPNL), kcal/kg/min

	Lactating	Pregnant	NPNL
Lying down	.022 ± .003[a,b] (12)	.020 ± .002 (8)	.018 ± .001[b] (3)
Making tortillas	.040 ± .007 (28)	.037 ± .008 (11)	.036 ± .007 (8)
Sweeping	.059 ± .010 (16)	.055 ± .011 (12)	.055 ± .005 (5)

[a] Mean ± standard deviation. Number of women in parenthesis

[b] Mean values differ, $p < 0.05$

be expected, tasks related mainly to agricultural activities required more energy than those related to child care and household maintenance.

The energy costs per kg body weight of activities measured in several pregnant, lactating and non-pregnant, non-lactating women did not differ (Table 3). This was probably due to the fact that there were few or no changes in these women's patterns of activities related to their physiological status (10) and their fitness and efficiency to perform routine tasks remained unchanged. The contribution of an increased basal metabolic rate in the last stages of pregnancy was not detected in the energy expenditure measurements. Pregnant women, however, do expend more energy per unit of time in those activities which require body displacement due to their greater weight.

The mean coefficient of variation of the 17 activities that were measured in 4 or more women was 22% (range 13-40%). The variability did not correlate with the number of observations nor with the energy expended in each activity, although the "light" occupations tended to have smaller variabilities. This could be, at least in part, because most activities entailed only one action (e.g., lying down, standing still) or a few actions which involved similar physical effort (e.g., sitting or sewing, picking coffee, winnowing).

The energy cost of carrying a load on a flat terrain or downhill, expressed per kg body weight, did not seem to differ from walking without the load (Student's "t" = 0.691). However, the total weight displaced by a 55-kg woman becomes 60 or 65 kg when she carries a 5- or 10-kg load, respectively, corresponding to an energy expenditure of 2.98 or 3.22 kcal/min compared to the 2.73 kcal/min that she would expend when walking without a load. More energy was expended per kg of body weight walking uphill with than without a load (Student's "t" = 2.164, $p < 0.05$) and the difference becomes greater when the total mass displaced is 60 or 65 kg (5.33 or 5.77 kcal/min, respectively) instead of 55 kg (4.25 kcal/min).

Table 4 compares the present study with similar or equivalent activities evaluated in other parts of the world. The results are usually similar and, with a few exceptions, the mean values reported by other investigators fall within the ranges shown in Table 1. The differences can be explained by the small number of measurements (sometimes made on one woman only), difference in methods, environmental conditions and inadequate description or lack of standardization of the activities involved. The latter can account for the large array of energy costs of washing clothes (type of clothes, body position, intensity of scrubbing), walking (gait and speed, inclinations and other characteristics of the terrain) and carrying a load (weight and volume of the load, how it is carried and the variables mentioned for walking), and for the discrepancies in many agricultural tasks (weight and type of rhythm, technique, inclusion of several actions or pauses within an activity, and so on).

The values shown in Table 1 can be applied to time-motion determinations in most of rural Central America and in other regions where the activities performed are similar to those described in the Appendix. The descriptions of the tasks in the references cited in Table 4 might apply better to other situations. As an alternative, the weighted averages shown in the last column of Table 4 can be used when the observed activities do not exactly coincide with those described in the sources for that Table. The data of Banerjee et al. (13) were not used to calculate the weighted averages, since they were usually lower than all others and the large number of observations would weigh markedly on the average. Data appended in the FAO/WHO 1973 report (1) are shown for comparison.

TABLE 4

Energy Cost of Equivalent Activities Performed by Women in Various Parts of the World

(kcal/kg/min)

	Present study	Machiguenga, Perú(4)	Nuñoa, Perú(5)	New Guinea (6)	China (11)	Various countries (12)	Negev, Israel (7)	Malaysia (13)	Marikina, Philippines (14)	Glasgow, Scotland (15)	Glasgow, older women (16)	Weighted mean[a]	FAO/WHO ref. woman (1)
Setting	rural	rural	rural	rural	rural	rural	rural	urban	urban	urban	urban		
Altitude, m	616	700	3950	coastal and highland		varied	150	20	20				
Age, years	16-49	16-41	20-66	18-49		young adult	20-30	18-42*	26-50	young adult	54-66	36	20-39
Occupation	H, A[b]	H, A	H, A	H, A	H, A	H, A	H, A	H	H	H.	H		
Mean weight, kg	49.1	43.3	52.0	48.6			55.7	53.4*	54.0	60.0	60.6	50.6	55.0
Women studied	56	4	8	78			8	79	10	4	17		
Lying down	.021(23)[c]		.021(8)					.018(79)*				.021	.016
Standing	.021(8)		.023(8)					.021(79)*	.024(10)	.023(8)		.023	.025
Sitting/sewing	.022(20)		.023(8)	.025(3)	.026	.024(5)	.024(2)	.019(79)*	.023(10)	.018(8)	.022(1)	.022	.021
Ironing	.026(1)							.030(79)*			.036(1)	.031	
Picking coffee	.027(6)		.033(8)	.061(10)[d]*								.030	
Washing dishes	.030(1)									.049(8)	.041(17)	.043	
Cooking	.032(19)						.036(1)		.036(10)	.042(8)	.038(17)	.036	.031
Housecleaning	.040(16)						.045(3)					.041	
Caring for children	.040(4)								.038(8)			.039	
Weeding	.047	.055(2)	.040(8)	.049(20)	.056	.064(1)	.060(2)					.048	
Washing clothes	.049(16)	.060(3)		.050(3)		.047(3)		.032(79)*	.044(3)	.051(8)		.050	
Walking on flat terrain	.050(31)		.055(8)	.054(13)	.059		.074(2)	.031(79)*	.046(10)	.075(8)		.054	.055
Tending animals	.050						.056(1)					.053	
Sweeping	.057(33)	.065(1)		.046(7)		.038(5)					.055(17)	.054	
Light work with machete	.064			.065(3)	.053							.062	
Gleaning	.072(5)	.077(2)				.070(1)						.073	
Lifting and moving objects	.074(4)		.085(8)		.087		.081(2)					.082	
Walking uphill	.077(18)			.101(17)								.089	
Chopping wood	.078(6)		.071(16)									.073	.069 - .180
Carrying a load uphill[e]	.089(24)			.109(10)								.095	

[a] When n was not available, a weight of 1 was given. Data with * were not used to calculate the weighted mean. [b] H = household activities; A = agricultural chores.
[c] Mean and number of measurements, when available. [d] Working on steep terrain. [e] The load's weight must be added to the woman's weight to calculate energy expenditure.

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APPENDIX

Energy expenditure was measured or estimated for the following activities and tasks, listed in alphabetical order:

Buying and selling: carrying out negotiations while standing; it often included walking a few steps.

Carrying loads: on the head, with the arms, or both; energy differences in the manner of carrying the load were minimal and values were therefore combined. The weight carried was added to the woman's weight to calculate energy expenditure per kg of body weight.

Child care: bathing, diapering, and dressing children and helping them to move about.

Chopping wood: wood 2-4 inches in diameter and dried tree branches were chopped with a machete (long-blade knife weighing 2-3 pounds); only the actual cost of chopping was measured.

Cooking: over wood fires using virtually no labor-saving devices. Typical activities included cutting of vegetables or meat; preparing and cooking rice, beans or corn (which entailed walking within the house to get the grain, walking to an outside faucet to draw water, apportioning grain or water, carrying the pot to the fire, stoking and poking the fire; and cleaning counters or a table. Environmental temperature in the kitchen was usually 27°C or higher.

Cutting fruit with a pole: a long pole was lifted and swung at fruits hanging from high branches. The woman then picked the fallen fruits from the ground and placed them in a basket that was carried to the next tree.

Eating: seated at a table the majority of the time, occasionally getting up to serve food to family members.

Gleaning: encompassed several activities: gathering wild fruit in the woods, scavenging fallen coffee berries for the household and gleaning grains after thrashing rice, sorghum or beans. This activity also included the picking of low plants or chili peppers and involved frequent crouching.

Hanging clothes: draping wet clothes over a line, bushes or on rooftops and removing them when dry.

Housecleaning: this is a general category for household duties that

account for a large portion of the women's work day. It included dusting, folding clothes, shaking out straw sleeping mats, collecting clothes for washing and "putting things in order". It can be considered as "random standing", as more standing than walking was involved.

Ironing clothes: was performed while standing, using a coal-filled or coal-heated iron.

Lifting and moving: activities such as stacking firewood, placing beans to dry under the sun and moving furniture and other household objects.

Light work with a machete: cutting low brush, usually to clear a trail while walking through fields.

Lying down: energy expenditure was measured after 15 minutes in this position.

Making tortillas: included kneading of corn dough and adding water until smooth; rolling the dough on a grinding stone; patting a small amount of dough with both hands into a round, thin pancake; tending the tortillas while they cooked on a griddle, and poking and stoking the fire. The grinding stones and fire were often located on opposite sides of the room and the process included frequent walking across the kitchen.

Nursing: usually while seated. When performing simultaneously other activities such as cooking or housecleaning, the energy cost of the most demanding activity was used to calculate energy expenditure.

Personal necessities: dressing and undressing, hair-combing, bathing and walking to an "out-house" to urinate or defecate.

Picking coffee: coffee berries were picked from branches within arm's reach of women standing beside the trees. Berries were placed into a basket tied to the women's waists and carried from tree to tree until the basket was full. The contents of full baskets (about 35 lb) were dumped into a gunny sack. Generally, coffee plantations were located on fairly flat terrain. The measurements made in this study coincided with a poor coffee harvest; during good seasons coffee is picked faster and in larger amounts per working hour.

Picking fruits: fruits, nuts, and leaves from bushes and trees within reach were picked while standing.

Raking and burning: raking leaves, grass, litter and trash into a heap for burning.

Sitting and sewing: these activities were combined, as women sewed while seated and energy expenditure was similar to that of non-activity.

Standing: either unsupported or while leaning against a wall.

Staking a fence: cutting saplings with a machete, digging holes with a stick, securing poles into the ground and tying fence wires around the poles.

Sweeping: with a broom. Dirt floors of the houses were sprinkled with water prior to sweeping.

Tending animals: herding pigs and cows short distances; preparing a slop

of corn dough, water, and salt and dropping it into a trough, and scattering grain for fowl.

Walking: was measured on local terrain, while ascending and descending mild slopes or on a flat surface. Generally, the women walked at a pace of about 4 km per hour.

Washing dishes: included lifting full buckets of water and vigorous scrubbing with coarse sand to remove grease and particles from pans and dishes.

Washing clothes: by hand either in a cement sink with a textured scrubbing surface or at a river. The rinsing of clothes was performed at the sink by scooping water and pouring it over the soaped clothes, whereas at the river clothing was dunked several times into the current. The women worked less continuously at the sink to allow the re-filling of the reservoir. Nevertheless, there was no difference in the amounts of energy expended using either method. Dirt was removed from the clothes by the women's physical force and weight against the roughness of the sink's surface or the rocks at the river; virtually no detergent and only mild vegetable soaps were used. The procedure consisted of wetting the clothes, soaping, scrubbing (anchoring the cloth with one hand and scrubbing with a back-and-forth motion using the other), rinsing and wringing; with heavy or exceptionally dirty clothing, both hands were used to scrub and the procedure was akin to kneading a tough piece of dough.

Weeding: stooping to cut weeds with a small curved knife, between bean plants or rows of corn.

Winnowing and other grain preparation performed while seated. This included shucking corn and removing dried kernels, cleaning undesirable particles from the grain, shaking or blowing grains to remove extraneous matter, shelling beans, separating grains for planting from those for eating and winnowing by pouring the grain into the wind for cleaning.

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THE SEQUENCE OF RECOVERY OF THE COMPLEMENT SYSTEMS AND PHYTOHEMAGGLUTININ SKIN REACTIVITY IN MALNUTRITION *

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ABSTRACT

Complement activities of the classical (CH50) and the alternate (ACH50) pathways, individual complement components and skin response to phytohemagglutinin (PHA) were studied in 51 malnourished children in Guatemala. PHA reactivity was significantly depressed in all malnourished children, particularly those who were less than 60% on the weight-for-height classification. Complement C3 and Factor B were significantly lower. On nutritional rehabilitation, the recovery of PHA reactivity was slower than recovery of the complement system.

KEY WORDS: Complement system, complement components, phytohemagglutinin reactivity, malnutrition, nutritional treatment.

INTRODUCTION

Cell-mediated immunity, phagocyte function and complement system have been shown to be impaired in the malnourished state (1-9). Many investigators have emphasized the consistent and early depression of cell-mediated immunity. The complement system is one of the major components of defense against infection. Complement C3 levels are significantly lowered in malnourished children (3,5, 7,11,12), even in those with marginal malnutrition (13). The individual components of the immune system are closely related to each other for the protection of the host against invading pathogens. We have been investigating the dynamic interplay between various immunologic components, especially cell-mediated immunity (CMI) and the complement system in malignancies and infections (14,15). Complement C3 plays an important part in linking humoral and cell-mediated immunity system (16). In our animal experiments (17), rats suffering from malnutrition showed negative tuberculin skin reactivity and the complement activity was reduced. After nutritional rehabilitation, the complement level recovered earlier than the delayed hypersensitivity reaction. The simultaneous estimation of complement activity and CMI in malnourished patients followed up during recovery is reported in this paper.

*This study was done at the Institute of Nutrition of Central America and Panama and was supported in part by the United Nations University, Programme of Nutrition and Infection of the Division of Biology and Human Nutrition.

MATERIALS AND METHODS

Patients

Fifty-one children, 6-36 months of age, with varying grades of malnutrition were examined. Eight were followed up for 3 weeks. In the follow-up study, all the items were examined on day 0 (the day before start of nutritional treatment), 7, 14 and 21 after the nutritional treatment. It may be pointed out, however, that these children were treated with antibiotics for a few days to control infection and had been on routine hospital diet during this initial period. All the children were hospitalized in the San Juan de Dios General Hospital of Guatemala city.

As controls, ten healthy, age-matched children clinically free from obvious infectious diseases were tested.

Nutritional status and infection

Anthropometric observations included measurement of height, weight, skinfold thickness over the triceps, scapula and abdomen.

Clinical manifestations examined included apathy, anorexia, skin changes (pigmentation, ulceration, desquamation and xerosis), hair changes (loss, dryness and depigmentation), edema, dehydration, hepatomegaly and cheilosis were noted.

Hematological observations included number of red blood cells and white blood cells, hemoglobin, hematocrit by Royco Cellcrit 920-A, albumin by the bromocresol purple mothod (18), and total plasma protein by refractometry.

Microbiological cultures of the throat, blood, urine and feces were done.

Phytohemagglutinin (PHA) skin reactivity

As an indicator of non-specific cell-mediated immunologic competence, PHA skin reactivity (19-21) was tested. PHA at a concentration of 0.1μg/0.1ml in sterilized isotonic saline (phytohemagglutinin M/P, Difco) was injected intradermally and the diameter of induration was read 24h after injection. The maximum vertical and horizontal diameters of induration were measured and the mean used for analyzing the results. Erythema also was observed but not employed for analysis. Skin tests were carried out by the same person throughout the experiment.

Complement activity and concentrations of individual components

For hemolysis through the classical pathway, CH50, a 50% hemolysis unit of sheep erythrocyte (SRBC) sensitized with rabbit antibody to SRBC by test serum was measured by Mayer's method (22). For the alternate pathway, ACH50, a 50% hemolytic unit of rabbit erythrocytes by test serum was measured (23). The individual components were determined by the single radial immunodiffusion method, using monospecific antisera to C4, C3 (Behring) and C1, C5, C9 and Factor B (prepared in our laboratory)

Heparinized blood samples were taken from the jugular vein and centrifuged at 100 g at 4°C. The supernatants were stored at -90°C until titration.

Statistics

The significance between the means for grouped data was calculated by Student's t-test.

RESULTS

Clinical observations

The patients were examined after antibiotic treatment for infection was over. The following clinical features were observed on the day of test i.e., edema in 6 patients, hair loss in 1, anorexia in 3 and apathy in 4.

Five of the subjects had urinary infection with Klebsiella and Pseudomonas. In 4 cases, *E.coli* and Proteus were found in the gastrointestinal tract. The bacterial counts were less than 10^5/ml. No sign of inflammation was observed in these patients.

In the patients who were followed up, the following features were observed on admission: Among the five children with marasmus, 4 showed apathy, 2 anorexia and 2 hair loss. Of the three patients with edema, 3 showed skin dryness and 1 anorexia.

PHA reactivity and complement levels

The nutritional status (Table 1) was classified according to the weight-for-height ratio. In this analysis, edematous patients were excluded. The diameter was bigger in the 81-100% weight-for-height, compared with the <60% group ($p < 0.001$). The proportion of individuals showing a positive response was 100% in the 81-100% group, 50% in the 61-80% group and 44% in the <60% group.

The CH50 titer did not differ in the three nutritional groups. Among the individual components, C4, C3 and Factor B were lower ($p < 0.05$) in the <60% group compared with the 81-100% group. Other components, C1, C5 and C9 did not show any significant differences among the three groups. The ACH50 titer was significantly lower in both malnourished groups.

Effects of nutritional treatment

The severely malnourished children were treated initially with 0.8g protein/kg/day and 100Kcal/kg/day and on day 14 and day 21 with 4g protein/kg/day and 100Kcal/kg/day. Eight children were observed for 3 weeks during treatment. All the parameters were measured on days 0, 7, 14 and 21 after initiation of nutritional supplements. The results of complement titers and levels of the individual components were summarized in Table 2.

Total protein, ACH50, C3 and PHA reactivity were significantly lower on admission. By the end of the first week of nutritional rehabilitation, CH50 and C3 had been restored to the normal level or higher. Total protein was restored by day 14 and ACH50 was restored more gradually. PHA reactivity showed slower recovery, the proportion of positive was 38% on admission, 50% on day 7, 63% on day 14 and 88% on day 21. Twelve percent were still negative after 3 weeks of nutritional treatment.

Changes in the complment level and PHA reactivity during nutritional recovery are shown in Fig. 1 and 2. Five out of 8 patients was negative to PHA on admission. Among five negative responders, one changed to positive on day 7, one on day 14 and two on day 21 after nutritional treatment was started. Three

TABLE 1.

Complement and phytohemagglutinin reactivity according to weight-for-height classification in malnourished children [a]

weight-for-height		60% n=11	p [b]	61-80% n=18	p [b]	81-100% n=16
PHA diameter	(cm)	0.8 ± 0.28	<0.001	1.1 ±0.34	NS	2.9 ± 0.28
PHA positive	(%)	44		50		100
Complement activity	(CH50)	51.5 ± 4.6	NS	53.4 ± 3.9	NS	54.4 ± 2.9
	(ACH50)	37.3 ± 2.3	<0.05	38.2 ± 2.3	<0.05	45.3 ± 1.8
C1	(c)	0.97 ± 0.02	NS	0.96 ± 0.02	NS	1.02 ± 0.01
C4	(mg/100ml)	23.1 ± 0.8	<0.05	25.3 ± 1.4	NS	31.7 ± 3.0
C3	(mg/100ml)	67.7 ± 4.5	<0.05	81.1 ± 4.2	NS	78.7 ± 4.1
C5	(c)	1.10 ± 0.02	NS	1.02 ± 0.01	NS	1.03 ± 0.02
C9	(c)	1.05 ± 0.03	NS	1.03 ± 0.03	NS	1.02 ± 0.03
Factor B		0.98 ± 0.03	<0.05	0.99 ± 0.03	NS	1.09 ± 0.02

[a] Edematous patients were excluded (6 cases)
[b] Significant difference from 81-100% NS: no significance
[c] Ratio:versus value in normal children
Numbers expressed Mean ± SEM

TABLE 2.
Total protein and complement in follow up study

Follow up days		day (n=8) 0	7	14	21	control	remarks
Total protein	(g/100ml)	6.23 ± 0.57**	6.84 ± 0.36*	7.41 ± 0.24	7.40 ± 0.14	7.66 ± 0.15	gradual
Complement activity	(CH50) [a]	46.0 ± 2.25	58.8 ± 4.19	61.0 ± 3.04	57.3 ± 3.77	52.4 ± 3.00	remarkable
	(ACH50) [b]	32.9 ± 1.38**	37.4 ± 1.29*	43.4 ± 3.13	44.5 ± 3.61	45.2 ± 2.13	gradual
C1	(c)	0.97 ± 0.02	0.97 ± 0.02	0.99 ± 0.02	0.97 ± 0.02	1.01 ± 0.01	no change
C4	(mg/100ml)	23.3 ± 1.55	24.3 ± 1.53	24.6 ± 1.53	26.5 ± 3.67	25.5 ± 0.78	gradual
C3	(mg/100ml)	70.5 ± 6.29*	76.4 ± 5.69	75.4 ± 2.90	80.3 ± 8.13	74.6 ± 0.91	remarkable
C5	(c)	1.01 ± 0.02	1.03 ± 0.03	1.02 ± 0.02	1.02 ± 0.03	1.00 ± 0.01	no change
C9	(c)	1.04 ± 0.03	1.07 ± 0.03	1.04 ± 0.04	1.04 ± 0.06	1.00 ± 0.02	no change
Factor B		0.98 ± 0.03	1.04 ± 0.03	1.05 ± 0.03	1.04 ± 0.05	1.01 ± 0.01	gradual
PHA reactivity diameter	(cm)	0.81 ± 0.41**	0.94 ± 0.39**	1.50 ± 0.51	2.03 ± 0.48	2.90 ± 0.31	gradual
positive ratio	(%)	38	50	63	88	100	gradual

* Significant difference from control $p < 0.05$, ** $p < 0.01$

Numbers expressed Mean ± SEM
[a] CH50 expressed 50% hemolytic activity of the sheep erythrocyte by the serum complement
[b] ACH50 expressed 50% hemolytic activity of the rabbit erythrocyte by the serum complement
[c] Ratio: versus control

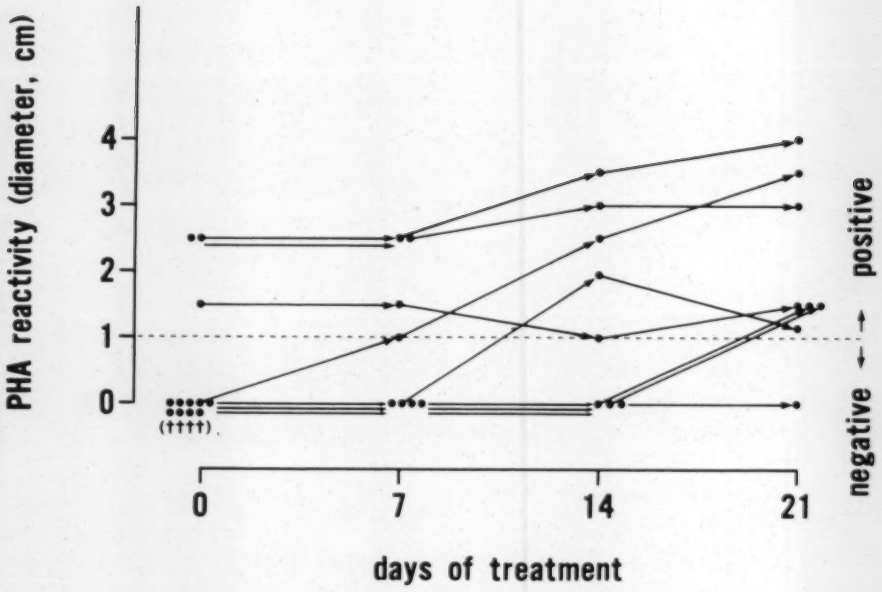

FIG. 1

PHA reactivity during nutritional recovery.

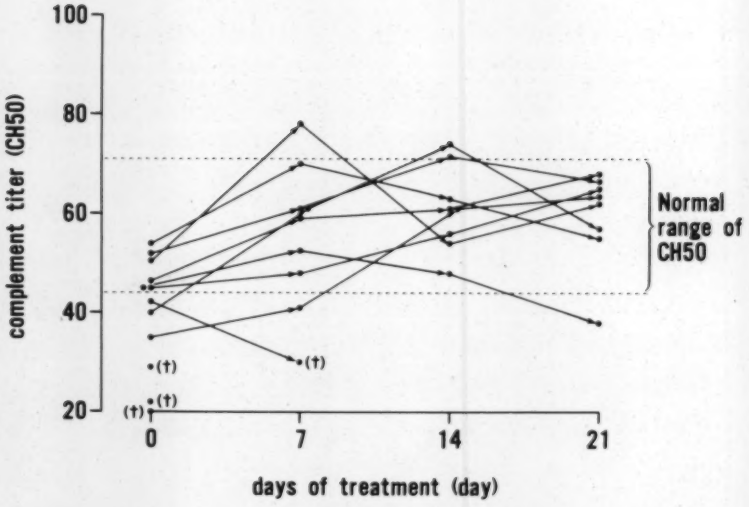

FIG. 2

Complement hemolytic activity during follow-up.
(†) symbolizes death.

with positive reactivity to PHA on admission gradually showed further increase in skin induration during the three weeks of follow-up.

On the other hand, in these 8 patients, the complement level was low but the mean was within the normal range on admission. Two cases were below the normal range. In all cases, the complement titer rose on day 7 and even further in the following days. Complement titer responded more quickly and earlier than PHA reactivity during nutritional treatment.

Four children out of the 12 initially included in follow-up died due to pneumonia and chicken pox during the early phase of the study period.

DISCUSSION

Reduction in hemolytic complement activity and individual components, specially C3, in malnourished children have been demonstrated by many investigators (5,11,12). Delayed cutaneous hypersensitivity and lymphocyte transformation are also depressed (2,3,10). It is clear that both the complement system and CMI quickly return to normal as a result of nutritional rehabilitation. Coovadia et al. (24) reported that the complement system returned to normal by the 10th day in 95% of malnourished children but PHA-stimulated lymphocyte transformation returned to normal on the 10th day in only 16.6% and on the 30th day in 60%.

Most of our subject suffered from infectious diseases on admission. The complement titers are influenced by infection as well as malnutrition. In this study, all the subjects were examined after antibiotic treatment for infection was over. CH50, C3, C4 and Factor B levels were low in the group below 60% in the weight-for-height classification. The diameter of induration of PHA reactivity was significantly smaller; the proportion of responders was 44%. Less prominent but nevertheless statistically significant changes were observed in mild malnutrition. PHA reactivity correlation with nutritional status. All the nutritional parameters were significantly lower in the PHA negative stage. When PHA response became positive, the total protein concentration returned to normal. In the PHA negative stage, ACH50 and Factor B were significantly lower. After PHA response turned to positive, C3 concentration showed a remarkable rise.

In the group of 8 who were followed up, on admission, the complement titer was lower in the PHA negative group. Following nutritional treatment, CH50 rose and later on PHA reactivity changed to positive and increased gradually. The CH50 and C3 levels recovered remarkably one week after admission but the PHA response recovered more slowly and after 3 weeks of treatment, 12% of the cases were still negative. Humoral immunity, including complement, can work in host defense during the early stage of nutritional recovery in malnutrition. Also, C3 may play an effective role in the activation of T-lymphocyte and macrophage cytotoxicities (25).

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IMPACT OF LONG TERM, LOW DOSE B-COMPLEX VITAMIN SUPPLEMENTS ON VITAMIN STATUS AND PSYCHOMOTOR PERFORMANCE OF RURAL SCHOOL BOYS

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ABSTRACT

Rural school boys having a very high incidence of biochemical vitamin deficiencies, were supplemented with recommended daily requirement of B-complex vitamins or placebo, for 1 year. The objective was to use the data for deriving the interpretive guidelines for biochemical tests of vitamin nutrition status. The supplemented children had significantly superior biochemical status of riboflavin, pyridoxine and folic acid as judged by erythrocyte glutathione reductase and erythrocyte aspartate aminotransferase activation coefficients, and RBC folate levels respectively. However, even in the supplemented group there was a wide scatter in biochemical values, precluding the use of the data for deriving interpretive guidelines. More than 50 percent of the boys in this group continued to have biochemical evidence of deficiency of one or more vitamins using reported criteria. However, selective psychomotor testing revealed beneficial effects of supplements on arm-hand steadiness test. Thus, functional impact of vitamin supplements may be seen even in the absence of clear-cut clinical or biochemical change.

Key words: B-complex vitamins, Rural children, Riboflavin, Pyridoxine, Folate, Vitamin A, Psychomotor performance.

INTRODUCTION

In a recent study on rural school boys we observed that all the boys suffered from biochemical evidence of riboflavin deficiency as judged by the erythrocyte glutathione reductase (EC1.6.4.2) (EGR) test. The incidence of pyridoxine deficiency as judged by the erythrocyte aspartate aminotransferase (EC 2.6.1.1) (EAspAT) test was also high (1). Treatment with B-complex vitamins containing 4mg riboflavin and 10mg pyridoxine daily for 1 month produced significant improvement in the saturation of both the enzymes with regard to their coenzymes, but almost 50 percent of the children continued to be in the deficient range, according to the guidelines provided in the literature for interpreting activation coefficients (AC) of enyzmatic tests (2). This would either mean that the interpretive guidelines suggested for discriminating between deficiency and sufficiency of vitamins B_2 and B_6, using enzymatic tests needed to be reconsidered, or that in this rural community vitamin supplements given over a period of one month, cannot achieve the expected level of tissue saturation for some unexplained reasons.

A study was, therefore, designed to find out if supplementation with B-complex vitamins at levels close to the recommended daily allowance (RDA) for a prolonged period of 1 year would achieve the desired degree of tissue saturation in the majority of the children, and to use this information for deriving guidelines for interpreting biochemical tests. The impact of vitamin supplements on growth, nutritional deficiency signs and symptoms and select parameters of psychomotor performance was also assessed.

Clinical and anthropometric examinations were done initially and then after 4, 8 and 12 months of treatment, on 200 children who were supplemented and 200 children given placebo. The data failed to reveal beneficial effects of vitamin supplements on growth, prevalence of nutritional deficiency signs or point prevalence of common ailments (3). The results of biochemical and psychomotor examinations done on a randomly selected subsample of 56 supplemented and 58 control boys, at the end of the study are reported here.

MATERIALS AND METHODS

School boys aged 5-11 years attending five rural schools within 25 km of Hyderabad were examined clinically and anthropometrically and divided evenly into supplemented and placebo groups, matching the two groups for age, anthropometry and presence or absence of nutritional deficiency signs and symptoms (3). Boys in the supplemented group received daily one tablet of B-complex vitamins containing thiamin 1mg, riboflavin 1.5 mg, pyridoxine 1.5 mg, niacin 15 mg, folic acid 0.1 mg and vitamin B_{12} 0.001 mg. Boys in the placebo group received a similar looking tablet containing 100 mg lactose. The two tablets were coded and the distribution was done by a school teacher who kept meticulous records of the tablet consumed by each boy. The tablets were analysed for riboflavin and pryidoxine contents at intervals of four months to ensure the stability of vitamins during storage. Since the teacher resided in the same village, he could distribute the tablets during holidays as well. As a marker of tablet consumption by the boys, early morning voided samples of urine were collected from 15 randomly selected boys from each group, supplemented or placebo, at each round of investigation and analysed for urinary riboflavin, by the microbiological assay using *L.casei* as the test organism (4).

The study was initiated in March, 1979. Clinical and anthropometric examinations were done initially and after 4, 8 and 12 months of treatment on 200 boys in each group. History of recent illness was elicited at each of the follow-up visits. At the end of the study, biochemical and psychomotor examinations were done a subsample of 56 and 58 boys from supplemented and placebo groups respectively. The subsamples from the two groups were matched for age and anthropometry. Only boys who had consumed the tablets for more than 80 percent of the days were selected for these investigations.

Samples of venous blood were collected and analysed for erythrocyte glutathione reductase activity (EC 1.6.4.2) (EGR) and its *in vitro* stimulation with FAD (EGR-AC) (5), for riboflavin status; erythrocyte aspartate aminotransferase activity (EC 2.6.1.1) (EAspAT) and its in vitro stimulation with pyridoxal phosphate (EAspAT-AC) (6) for pyridoxine status. RBC folate concentration was measured by the microbiological assay using *L.casei* as the test organism (4). Plasma vitamin A levels were assayed by the spectrophotometric method (7). Haemoglobin was estimated by the cyanmethaemoglobin method. Examination of thiamin status was not done in the present study because the earlier study had shown that thiamin deficiency is not a problem in this community. This may be due to associated calorie deficiency (1).

The psychological testing was done using the following 4 psychomotor and mental performance tests: Multichoice Reaction Time to Visual Stimulus (8), Finger Dexterity Test (8), Arm Hand Steadiness Test (8) and Knox Cube Imitation Test (9). Due to practical difficulties only these four tests could be applied. Their selection was based on prior established validity of the test to measure a particular ability (10) and prior evidence that a given measure would reflect aspects of specific vitamin deficiency (11).

Comparisons between the supplemented and the placebo groups were done by Student's t-test or modified t-test. Person's correlation coefficients between biochemical and psychomotor tests were calculated (12).

RESULTS

The children in the supplemented group had significantly lower EGR-AC and EAspAT-Ac values and higher RBC folate levels compared to the placebo group, indicating that the supplements were being utilised (Table 1). Urinary excretion of riboflavin was also significantly higher in the supplemented children (3). Vitamin A levels were similar in the supplemented and placebo groups, suggesting that B-complex citamin supplements do not affect vitamin A status (Table 1). Supplemented group had slightly lower mean value for haemoglobin.

TABLE 1

Riboflavin, pyridoxine and folic acid status of vitamins supplemented and placebo groups

Parameters	Treatment	
	Placebo (n=58)	Supplement (n=56)
EGR-Basal activity[a]	79.45 ± 2.167	107.57 ± 3.262***
EGR-AC	1.83 ± 0.037	1.43 ± 0.030***
EAspAT-Basal activity[b]	62.67 ± 2.093	76.86 ± 2.073
EAspAT-AC	1.86 ± 0.028	1.66 ± 0.028***
RBC Folic acid (mμg/l)	133.9 ± 5.63	187.8 ± 7.91 ***
Plasma vitamin A (μg/dl)	36.7 ± 1.65	33.7 ± 1.77
Haemoglobin g/dl	12.8 ± 0.13	12.2 ± 0.18 **

a μmol NADPH oxidized/ml erythrocytes per h.
b μmol NADH oxidized/ml erythrocytes per h.
Values are Mean ± SEM. ** $P < 0.01$, *** $P < 0.001$

Data in Table 2 show that in the placebo group, the incidence of high risk vitamins B_2, B_6 and folic acid deficiency was 88.8, 24.1 and 54.7 percent respectively. In the supplemented group the incidence of vitamin deficiencies in the high risk range was markedly reduced, but 49% continued to suffer from riboflavin deficiency and 21% from folic acid deficiency. After a full year of vitamin supplements, only 17.9% of the boys showed evidence of satisfactory

riboflavin status, the corresponding figures for pyridoxine and folic acid status being 55% and 65% respectively. B-complex supplements had no effect on plasma vitamin A levels.

TABLE 2

Percent distribution of boys according to biochemical status of riboflavin, pyridoxine and folate in supplemented and placebo groups

Treatment	Low Risk	Medium Risk	High Risk	
		EGR-AC		
	< 1.2	1.2 - 1.4	1.4 - 1.6	> 1.6
Placebo	0	5.2	15.5	79.3
Supplement	17.9	32.1	30.4	19.6
		EAspAT-AC		
	< 1.7	1.7 - 2.0		> 2.0
Placebo	29.3	46.6		24.1
Supplement	55.4	41.1		3.5
		RBC folate (mμg/ml)		
	> 160	140 - 160		< 140
Placebo	26.4	18.9		54.7
Supplement	65.1	14.0		20.9

The boys in the supplemented group had significantly lower error scores in the steadiness test compared to the placebo group, indicating better performance (Table 3). Their mental age also tended to be slightly higher but the difference was statistically not significant. A weak direct correlation ($r = 0.225$, $p \leq 0.05$) was observed between the steadiness test error scores and EGR-AC.

TABLE 3

Psychomotor performance of vitamin supplemented and placebo groups

Test	Treatment	
	Placebo (n=58)	Supplement (n=56)
Mental age (years)	9.76 ± 0.436[3]	10.89 ± 0.487
Steadiness test (error scores)	332.1 ± 17.32	246.0 ± 13.72 ***
Reaction time (seconds)	702.0 ± 18.78	706.7 ± 18.35
Finger dexterity test (seconds)	525.4 ± 25.85	500.0 ± 18.70

Values are given as Mean ± SEM
***P < 0.001

Date in Table 4 shows that among children whose error scores in the steadiness test were higher than 420, regardless of the treatment, 90.5% were deficient in riboflavin. In contrast, among children whose error scores were less than 170, only 60.9% were deficient. The difference in the distribution was significant (P < 0.05) indicating that children with superior riboflavin status tended to perform better in the Steadiness test.

TABLE 4

Relationship between riboflavin status and errors committed in steadiness test

Group[a]	Errors Committed	EGR-AC	
		< 1.4	> 1.4
1	< 170	39.1	60.9
2	170 - 420	28.2	71.8
3	≥ 420	9.5*	90.5*

* Significantly different from group 1 by proportion's test for small samples. P < 0.05.
a Values for both the treatment groups were pooled. Data are shown as percent distribution.

DISCUSSION

Diets of rural communities in India are known to be deficient in several vitamins. An earlier diet survey in this study population had shown that the diets of these children are markedly deficient in vitamins, particularly riboflavin (1). At the time of initiating this study it was hoped that prolonged intake of vitamins at levels exceeding the RDA, would produce optimum levels of tissue saturation, and reduce the scatter in biochemical values. The data were expected to furnish guidelines for discriminating between vitamin deficiency and sufficiency using biochemical tests. However, despite a markedly superior biochemical status of the supplemented children compared to the placebo group, the data could not be used for obtaining interpretive guidelines because of a large scatter in biochemical values in both the groups.

The reasons for persistent biochemical vitamin deficiency in so many children after ensuring optimum intake for a prolonged period of time are not clear. In the same study, a marked seasonal variation in the urinary excretion of riboflavin in the supplemented as well as placebo groups was observed (3). Very high urinary excretion of riboflavin was observed in winter. The incidence of respiratory infections was also higher in winter (3). It is possible that repeated bouts of infections, and dietary deficiency of calories and proteins would keep these children in a state of negative nitrogen balance. This would lead to urinary losses of vitamins and tissue depletion. Improvement in dietary intake of vitamins would fail to produce the expected magnitude of tissue saturation and clinical improvement under these environmental constraints. The reason for the lower mean value for haemoglobin in the supplemented group is not clear.

Though vitamin supplements did not produce the expected reduction in the incidence of nutritional deficiency, signs such as angular stomatitis and glossitis which are pathognomonic of B-complex vitamin deficiency (3), the supplemented children performed better in the steadiness test, a measure of psychomotor performance. This effect of vitamin supplements may be due to the riboflavin component, since a correlation between riboflavin status as judged by EGR-AC, and errors in steadiness test was apparent. Similar correlation was not seen with pyridoxine or folate status. Experimental riboflavin deficiency in humans has been reported to weaken the hand grip strength (13). Reduced steadiness of hands and weakened hand grip may both be due to decrease in the concentration of flavoporteins and oxidative phosphorylation in the muscle.

In conclusion, the results of the study reported earlier (3) and in this communication show that in some impoverished communities, it may not be possible to saturate the tissues with vitamins despite adequate dietary intake. This phenomenon may be related to metabolic losses of the vitamins. The data also suggested that a functional impact of vitamin supplements may be seen even in the absence of clear-cut clinical impact as judged by established nutritional deficiency signs.

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PLASMA LACTIC DEHYDROGENASE ELECTROPHORETIC PATTERNS OF LONG-TERM VITAMIN E-DEFICIENT RATS[1]

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ABSTRACT

Weanling male Sprague Dawley rats were fed a vitamin E-deficient basal diet with or without supplementation of 100 IU vitamin E per kg. diet for 12 months. Plasma total activity of lactic dehydrogenase (LDH) was significantly higher in the vitamin E-deficient group than the control group. Isoenzymes of LDH in plasma were separated by electrophoresis, and quantitated. The results showed the presence of five isozymes (LDH_1 - LHD_5) in the vitamin E-deficient plasma as compared with three isozymes (LDH_1, LDH_4 and LDH_5) in the control plasma. The LDH_1, LDH_4 and LDH_5 isozymes were significantly elevated in the vitamin E-deficient group when compared with the control group. The LDH_2 and LDH_3 isozymes, which were not observed in the electrophorogram of the control plasma, appeared in the electrophorogram of vitamin E-deficient plasma. The electrophorogram of LDH isozymes, combined with the results of a previous tissue enzymatic and histopathological study, suggests the tissue sources of LDH leakage into the blood in vitamin E deficiency are mainly heart (LDH_1, LDH_2, and LDH_3) and skeletal muscle (LDH_4 and LDH_5).

KEY WORDS: Lactic dehydrogenase isozyme, electrophoresis, vitamin E-deficiency

INTRODUCTION

Lactic dehydrogenase (LDH) (EC 1.1.1.27) catalyzes the interconversion of lactate and pyruvate with nicotinamide adenine dinucleotide as the cofactor. The activity of this enzyme can be detected in all tissue and can be identified with a tissue specified pattern of five isozymes in most

[1] The investigation reported in this paper (No. 81-9-16) is in connection with a project of the Kentucky Agricultural Experiment Station and is published with the approval of the Director.

mammals and birds. The random association of two different polypeptides, A and B, forms the five possible tetramers A_4, A_3B_1, A_2B_2, A_1B_3 and B_4 (1,2) which were designated as LDH_5, LDH_4, LDH_3, LDH_2, and LDH_1 later. Tissues normally subjected to periods of anaerobiosis, like skeletal muscle, are richer in A subunits, and tissues with a constant supply of oxygen are richer in B subunits (3,4).

In human sera, five isozymes of LDH are found. The determination of the activity of the isozymes of this enzyme in plasma or serum is utilized clinically in man as an aid to diagnose disease due to enzymes released into blood by damaged tissues. For example, the LDH_5 is increased in sera of patients manifesting hepatic necrosis, and LDH_1 is increased in sera from partients suffering from acute myocardial infarction (5).

In a previous study conducted in this laboratory, it was found that the total LDH activity increased significantly in the plasma of long-term vitamin E-deficient rats when compared with that of controls (6). The purpose of this study are to separate and quantitate the plasma LDH isozymes, and to find the tissue sources of LDH which is released into the plasma of rats with long-term vitamin E deficiency.

METHOD

Ten weanling male Sprague Dawley rats[2] were fed a vitamin E-deficient basal diet (7) with or without supplementation of 100 IU vitamin E per kg diet. The rats were killed after 12 months, blood samples were collected. The vitamin E status was assessed by the spontaneous hemolysis test of erythrocytes (8), and the determination of the plasma level of vitamin E (6). Plasma total activity of lactic dehydrogenase was determined by the method of Wroblewski and LaDue (9).

One male Sprague Dawley rat of body weight about 300 g was killed. The brain, kidney, heart, testis, spleen, liver and a portion of skeletal muscle from lower extremity was removed. Approximately 10% homogenate of each tissue was prepared with a solution containing 0.25 M sucrose and 0.05 M phosphate buffer (pH 7.4). After centrifuging at 1000 xg for 10 minutes, the supernatant was further centrifuged at 30,000 xg for 30 minutes. Proper dilution of each supernatant was made before application onto the cellulose polyacetate strip for electrophoresis.

Isoenzymes of lactic dehydrogenase were separated by the electrophoretic method of Preston et al. (10) using cellulose acetate strips (Sepraphore III support membrane[3]).

Cellulose acetate strips were soaked overnight in an electrophoretic buffer consisting of tris-sodium barbitol-barbitol (pH 8.8, ionic strength 0.03). The strips were blotted on a piece of blotting paper and 20 µl of each sample were applied as a streak 2.5 cm from the cathode end. Electrophoresis was conducted at room temperature at 300 volts for 45 minutes. The stain working solution containing sodium lactate, nicotinamide adenine dinucleotide, nitroblue tetrazolium and phenazine methosulfate was prepared near the completion of the time required for the electrophoretic separation. The substrate strip was prepared by soaking a clear dry cellulose acetate strip in the stain working solution, and it was placed on a glass microscopic slide. Immediately after the electrophoretic run, each strip was removed from the electrophoretic chamber and superimposed on a substrate strip placed in a moist chamber. The covered moist chamber was then incubated for 30 minutes at 37°C. The isozymes of LDH

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[2]Harland Laboratory Animals, Harland Industries Inc., Indianapolis, Indiana.

[3]Gelman Instrument Co., Ann Arbor, Michigan 48106.

appeared as distinct blue separated bands. The strip was than fixed in 1% formalin for 10 minutes and dried overnight between two absorbant sheets. The blue bands of the plasma electrophorogram were quantitated by densitometry. The areas under the curve were measured using a planimeter, and the percentage of each isozyme was calculated by dividing individual area with the sum of the areas. The activity of each plasma LDH isozyme was calculated by multiplying the total plasma LDH activity with percentage of each isozyme.

RESULTS

The results of spontaneous hemolysis test showed that the percent hemolysis of erythrocytes from vitamin E-deficient group and control group were 97.2 ± 1.1% and 8.7 ± 2.5% ($p<0.0001$), respectively. The plasma levels of vitamin E for the vitamin E-deficient group and control group were 36.9 ± 14.4 and 846.0 ± 96.1 µg/100 ml ($p< 0.0001$), respectively. These results clearly showed that the degree of vitamin E deficiency was quite severe in the deficient group.

Fig. 1 shows the illustration of the electrophoretic strips for the brain, skeletal muscle, kidney, heart, testis, spleen and liver. The LDH isozyme patterns of the seven selected tissues can be seen. The strips for the brain, kidney, heart and testis show five clear bands of all five isozymes of LDH. For the heart, LDH_1, LDH_2 and LDH_3 are the bigger bands. For the kidney, LDH_1 and LDH_2 are the bigger bands. For the testis and brain, LDH_5 is the smallest band, with the other four isozymes about equal size. The strip for the skeletal muscle shows the LDH_5 band as the biggest band, and LDH_4 band as the next biggest. Faint bands of LDH_1, LDH_2, and LDH_3 can also be seen. The strip for spleen shows big bands of LDH_5 and LDH_4, and a small band of LDH_3. The strip for liver shows a largest band for LDH_5 and a medium band of LDH_4 with a faint band of LDH_3.

Fig. 2 shows the scanning of one representative electrophoretic strip from each of the vitamin-E deficient and control groups. In vitamin E-deficiency, all five isozymes of LDH can be seen: LDH_1 is the second biggest peak which is closest to the anode end and LDH_5 is the biggest peak closest to the cathode end. In the control, the second biggest peak closest to the anode end again is LDH_1. The biggest peak closest to the cathode end is LDH_5. There is a very small band of LDH_4 next to LDH_5 in all the plasma samples from the control rats LDH_1, LDH_4 and LDH_5 were all markedly elevated in the vitamin E -deficient group when compared with the control group. LDH_2 and LDH_3, which were not found in the control, were observed in vitamin E-deficiency.

Table 1 shows the quantitation of each isozyme on the electrophoretic

TABLE 1
Activities of Plasma lactic dehydrogenase (LDH) isozymes in vitamin E-deficient and supplemented rats[1,2,3]

LDH Isozyme	Vitamin E Deficient	Vitamin E Supplemented	Level of Significance
LDH_1	100 ± 10 (20.8%)	29 ± 5 (15.6%)	P<0.001
LDH_2	23 ± 7 (4.8%)	0	P<0.0001
LDH_3	32 ± 10 (6.6%)	0	P<0.0001
LDH_4	56 ± 11 (11.6%)	5 ± 2 (2.7%)	P<0.0001
LDH_5	271 ± 37 (56.2%)	151 ± 9 (81.2%)	P<0.005
Total	483 ± 42 (100%)	186 ± 13 (100%)	P<0.001

[1]Mean ± SE of 5 rats. [2]Nmole NADH oxidized/ min/ml plasma.

[3]Data in the parenthesis show the percent of total LDH activity.

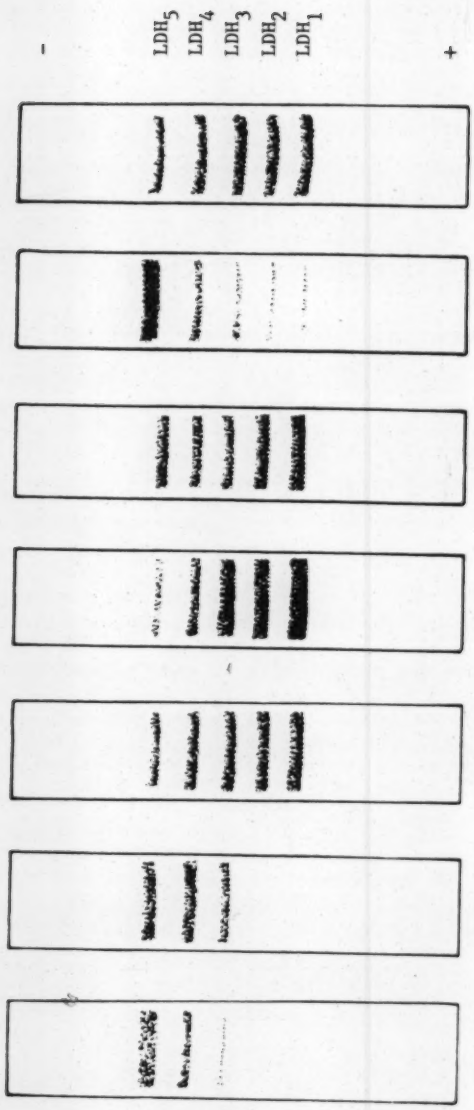

FIG. 1

Electrophoretic Patterns of Lactic Dehydrogenase (LDH) Isozymes of Selected Rat Tissues

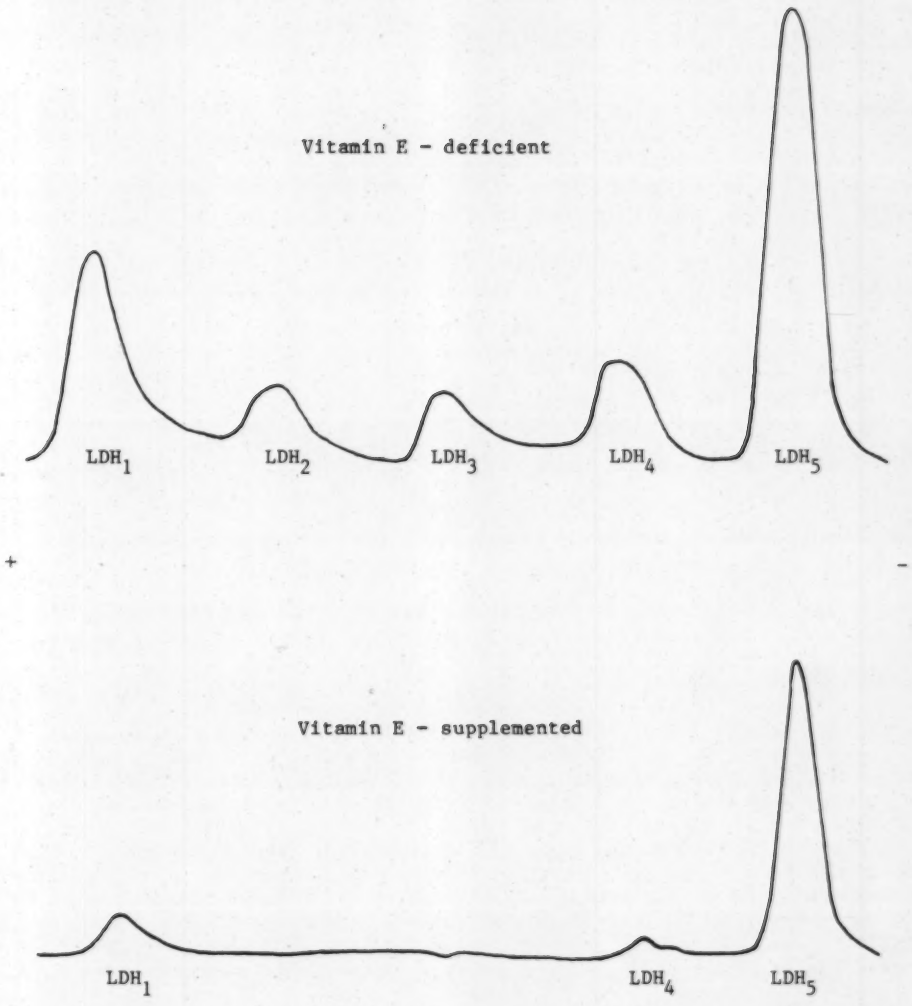

FIG. 2

Electrophorograms of Plasma Lactic Dehydrogenase (LDH) Isozymes of Vitamin E - Deficient and Supplemented Rats

strips of vitamin E-deficient and supplemented groups. The activity of LDH_1, LDH_4, and LDH_5 in the vitamin E-deficient group was elevated to 345% ($P<0.001$), 1120% ($P<0.0001$), and 179% ($P<0.005$) of the control group, respectively. The total LDH activity in the vitamin E-deficient group was elevated to 260% of the control group ($P<0.001$).

In the control plasma, LDH_5 was 81% of the total LDH activity, however, in the vitamin E-deficient plasma, LDH_5 activity was lowered to 56% of the total LDH activity due to the increase of all four other LDH isozymes. The percentage of LDH_1 activity was 16% and 21% in the supplemented and deficient group, respectively. The total LDH activity in the vitamin E-deficient group was elevated to 260% of the control group ($P<0.001$).

DISCUSSION

Nearly every tissue or organ of the adult rats or mice contains all five isozymes of LDH, but in proportions that are highly specific for the tissue (4,11,12,13). LDH_1 and LDH_2 are found chiefly in the heart and kidney, LDH_3 in the heart, LDH_4 and LDH_5 in the skeletal muscle, spleen and liver. The results of this study (Fig. 1) showed that LDH_1, LDH_2 and LDH_3 were found chiefly in the heart, kidney, brain and testis, LDH_4 was found in all seven tissues studied: the skeletal muscle, spleen, liver, brain, kidney, heart and testis. While LDH_5 was found chiefly in the skeletal muscle, spleen and liver.

In normal human sera, five isozymes of LDH are usually found (10). However, in this study only three isozymes: LDH_1 and LDH_5, with a trace of LDH_4, were observed in the control rat plasma. Garbus et al. (13) also observed three LDH isozyme bands after electrophoretic separation of rat serum.

The histopathological finding at the light microscopic level showed marked pathological changes in the skeletal muscle, heart and testis, but not in other tissues of vitamin E-deficient rats (6). In the same study, it was found that the LDH activity in the heart and skeletal muscle was significantly decreased in vitamin E deficiency when compared with the control, whereas the enzyme activity in the brain, lung, liver, kidney, spleen and testis was not significantly altered in vitamin E deficiency. By considering the above findings together with the electrophoretic pattern of LDH in this study, it becomes evident that the marked increase of LDH_1, LDH_2, and LDH_3 in the vitamin E-deficient plasma over the control plasma suggests the leakage of the enzyme from the heart. The marked increase of LDH_4 and LDH_5 in the vitamin E-deficient plasma over the control plasma suggests the leakage of the enzyme from the skeletal muscle.

The present study also confirmed the previous finding that total LDH activity increased significantly in the plasma of rats with 12-month vitamin E deficiency (6). However, it has been reported that the LDH activity in the plasma, erythrocytes, lung, liver, and muscle of rats with 4-month vitamin E deficiency were not significantly different from the control group (14). The different results in the above two studies can be explained by the difference in the feeding period. The leakage of LDH from the damaged tissues into blood occurs in the severe vitamin E deficiency but not in a mild vitamin E deficiency.

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EFFECTS OF A LIQUID PROTEIN DIET DURING PREGNANCY ON NEWBORN RATS

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ABSTRACT

Liquid protein was introduced in the mid 1970's as a means of weight control, and has been used extensively within the general population. The protein portion of the standard casein diet was substituted with liquid protein and fed to pregnant rats. The newborns whose mothers were fed liquid protein with all the other necessary nutrients showed an increased rate of stillbirths and cataracts. The total body weight was decreased compared to the pair-fed and/or ad libitum controls, whereas the organs such as the heart, lung, kidney and liver were smaller than ad libitum controls. The data suggest that taking liquid protein requires caution, particularly during pregnancy.

KEY WORDS: Liquid protein, newborn, growth and development

Since liquid protein diets (LPD) were introduced in the mid-1970's as a means of weight control, numerous deaths associated with these diets have been reported in clinical literature (1,2,3). It has been estimated that approximately 100,000 persons have used liquid protein as a means of weight control as their sole source of nourishment for at least one month (1). Until recently, no scientific studies using animal models had been conducted to show whether or not this dietary regimen was indeed safe (4). However, in 1979 FDA scientists completed studies to determine the growth-promoting quality of the protein-containing liquid protein foods in growing male rats. They found that the nutritional quality was poor in comparison to casein (3). To date, nothing has yet been done with pregnant animal models to show what effects may occur during growth and development. Although pregnant women are advised not to take liquid protein (3,5), this study was conducted because of the concern that women taking these products for weight control may become pregnant and not discover it for one to three months. The possibility also exists that some women may adopt this diet during pregnancy without informing their physician in order to control weight gain, with possible effects to the fetus. The present study was designed to investigate the effects occurring during growth and development in fetal rats whose mothers were given liquid protein as their sole source of protein, in addition to receiving all the other nutrients known to be necessary for normal growth. Furthermore, the experiment was designed to show the effects of liquid protein on fetal rats whose mothers were fed with this product during various time periods of pregnancy.

MATERIALS AND METHODS

A total of 29 timed-pregnant rats were used. Upon arrival, all were fed a standard stock diet until day nine or thirteen. These dates were arbitrarily chosen because of the concern that giving liquid protein in the very early stages might induce either abortion or resorption of the fetus and that the fetuses would have better chances for survival by starting around these times.

The dams were randomly divided into three major groups. This consisted of 19 rats being fed a 20% LPD, four being given a 20% casein diet ad libitum and six which were pair-fed with the liquid protein group, but which received a 20% casein diet rather than liquid protein.

The casein diet becomes slightly higher in energy and protein content on a gram per gram basis compared to the LPD. However, because of the different nature of the two diets, i.e. one is powdered and the other is semi-liquid, only a small adjustment in food intake for the pair-fed group with respect to energy and protein intake was necessary in order to approximate the food intake along these parameters.

The liquid protein group was further subdivided into two subgroups in order to evaluate the effects which might occur in newborns whose mothers received the liquid protein during several stages of pregnancy. Group 1a contained eleven pregnant rats which were fed liquid protein from day 13 until day 18 (experiment 1) and group 1b consisted of eight rats who received liquid protein from day 9 until day 19 (experiment 2).

The liquid protein was purchased from a health foods store and is commercially available across the United States as "liquid protein" (Vangard Products Liquid Protein Wild Cherry Flavor). The diet was based on previous research involving casein diets which were given to laboratory rats (6). This diet contained enough carbohydrates, fats, vitamins, and minerals for normal growth and development (Table 1). The liquid protein was substituted in lieu of casein as the protein component in the LPD. The remaining dietary components were identical in both diets. Thus, any detectable differences in fetal development could be attributed solely to the substitution of the protein component, without the added complication of possible effects resulting from malnutrition or induced deficiency states.

Because the rats fed liquid protein did not experience weight gain while on this diet as compared to the other groups, nineteen rats from subgroup 1a and 1b were returned to the normal 20% casein diet on day 18 and 19, respectively. Both the pair-fed and ad libitum groups remained on the 20% casein diet throughout the entire period. Food intake and weight changes in the pregnant females were recorded daily. Upon delivery, offspring were evaluated within eight hours for any detectable gross abnormalities and their weights recorded.

The gross abnormalities and anatomic defects seen in these preliminary studies were stillbirths, cataract formation, and differences in organ weights between the groups. Stillbirths were designated as those newborns found dead within eight hours after delivery. This definition was adopted because of the impossibility of observing every delivery occurring during the day or night. Cataract formation was defined as the presence of an opaque lens which was white in color. Specimens were sent to an ophthalmologist at the LSU Eye Center for confirmation.

For a relative comparison of the diets, the protein efficiency ratio during days 13 to 18 was calculated for the LPD and pair-fed groups. This was

TABLE 1

Composition of diets

	20% Casein (g)	20% Liquid Protein (g)
Casein	200	400* ml (200 g)
Dextrose	192	192
Sucrose	178	178
Dextrin	192	192
Mazola Corn Oil	150 ml	150 ml
Mineral Mix**	40	40
Choline Chloride (50% w/v)	4 ml	4 ml
Cellulose	35	35
Vitamin Mix***	10	10

*According to the label on the bottle, thirty milliliters of liquid protein correspond to 15 g of soluble protein hydrolysate and the amino acid composition is approximately the following in milligrams: L-Alanine, 1300; L-Arginine, 1200; L-Aspartic acid, 900; L-Cystine, 10; L-Glutamic acid, 1500; Glycine, 3500; L-Histidine, 70; L-Hydroxylysine, 150; L-Hydroxyproline, 2200; L-Leucine, 450; L-Isoleucine, 200; L-Lysine, 650; L-Methionine, 110; L-Phenylalanine, 350; L-Proline, 2300; L-Serine, 1000; L-Threonine, 300; L-Tryptophan, 65; L-Tyrosine, 100; L-Valine, 350. Although most of the amino acid concentration in the present diet is identical to prolinn (5), the concentration of two amino acids were different, i.e. L-Histidine 110 mg and L-Hydroxyproline, 1000 mg.

**Rogers-Harper mineral mix (Teklad Test Diets, Madison, WI.)

***AIN vitamin mixture 76 (ICN Pharmaceuticals, Inc. Cleveland, Ohio)

calculated by dividing the total average weight gain in the group during days 13 to 18 by the total average protein consumed during the same period.

After evaluation, randomly selected offspring from the LPD mothers, the pair-fed and ad libitum group of experiment 1 were sacrificed and various organs weighed. The data were analyzed using the analysis of variance and multiple comparison (the Student-Newman-Keuls) with a 5% level considered as statistically significant.

RESULTS

The offspring from the liquid protein groups consistently weighed less than the casein groups (Table 2 and Fig. 1). The hearts, tongues, mandibles and long bones showed no differences statistically. On the other hand, the lung, kidney and liver were smaller as compared to the ad libitum controls.

In general, the mothers did not gain weight while being fed the LPD during the various experimental periods as compared to pair-fed (Fig. 2,3) and/or ad libitum groups which continuously gained weight during the period (Fig. 4). However, upon returning to a 20% casein diet, there was a huge increase in body weight within 24 hours (Fig. 2,3) followed by a small increase or no change in weight. Food intake did not vary much from day to day when considering the standard deviation. (Subsequent studies indicate that there is a tendency to eat slightly less of the liquid protein diet as the experiments continue).

TABLE 2

Body and organ weights of newborn rats delivered to mothers fed LPD during days 13 to 18 of gestational age, and their controls

	Body Weight (g)	Heart (mg)	Lung (mg)	Kidney (mg)	Liver (mg)	Tongue (mg)	Mandible (mg)	Long Bone (mg)
LPD fed Exp. 1	* 5.60[c] ±0.90 **	24.7 ±2.9	91.2 ±9.3 **	20.5 ±6.4 **	243.6 ±54.5 **	56.0 ±10.3	18.1 ±5.3	12.8 ±3.1
Pair-fed[a] Exp. 1	* 6.12 ±0.48	25.9 ±4.5	** 94.8 ±9.5	* 22.9 ±1.1	* 261.6 ±41.3	52.2 ±7.9	18.2 ±2.5	11.6 ±1.3
Ad libitum[b]	6.77 ±0.55	29.0 ±3.0	115.6 ±10.1	29.6 ±5.2	361.9 ±75.5	64.4 ±12.4	20.3 ±4.8	15.3 ±5.6

[a]These animals were pair-fed with the LPD group while on the liquid protein, as well as when the LPD animals were given the 20% casein diet until birth. A slight adjustment of food was done.

[b]These animals were fed on ad libitum 20% casein diet from day 13 to 22.

[c]Means and standard deviation

Statistical significance *$p < 0.05$, **$p < 0.01$.

The asterisk within the LPD fed group shows the statistical significance with the pair-fed group, and that below the number shows the statistical significance with the ad libitum control group. The asterisk in pair-fed group shows the statistical significance with the ad libitum group.

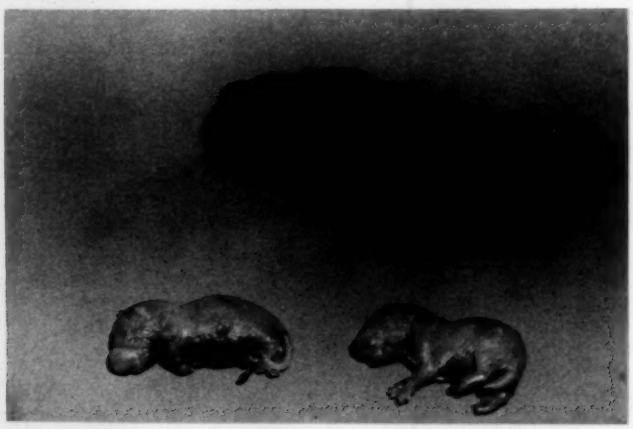

Fig. 1. At the top of the picture a newborn control is seen which was larger in gross size than those fed liquid protein. In the lower right a liquid protein newborn shows its smaller size compared with the control at the top. The newborn control could not be placed in the position the liquid protein offspring assumed, possibly indicating an inherent weakness in the liquid protein newborn at birth. In the lower left is a stillbirth offspring from an LPD group.

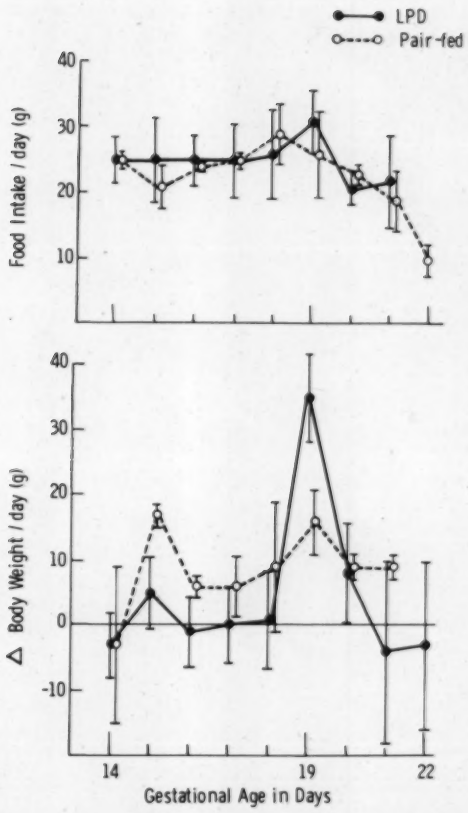

Fig. 2. Food intake and changes in body weight seen in mothers while being fed a 20% liquid protein diet between days 13 to 18. From day 18, the diets were changed to a 20% casein diet until birth. Pair-fed mothers received a 20% casein diet throughout the experimental period as compared to the 20% LPD group. Pair-fed animals on several occasions did not finish the food. When calculating the amount of food intake for a pair-fed sometimes an abnormally high or low food intake occurred in LPD group, necessitating elimination of that animal from the calculations to minimize the error. However, in the diagram there was no elimination of the abnormal food intake. This caused the huge standard deviation.

After averaging all the experimental groups together, stillbirths occurred 2.5 times as often in those rats fed liquid protein as compared with controls (Table 3). Cataract formation occurred exclusively in those newborns whose mothers received liquid protein during various stages of pregnancy. All of the liquid protein groups averaged together exhibited an incidence of 15%, whereas pair-fed and ad libitum groups did not show any sign of cataract formation (Table 3 and Fig. 5).

DISCUSSION

The liquid protein preparations are formulated from hydrolysates made largely from collagen or gelatin derived from cowhides and other sources of

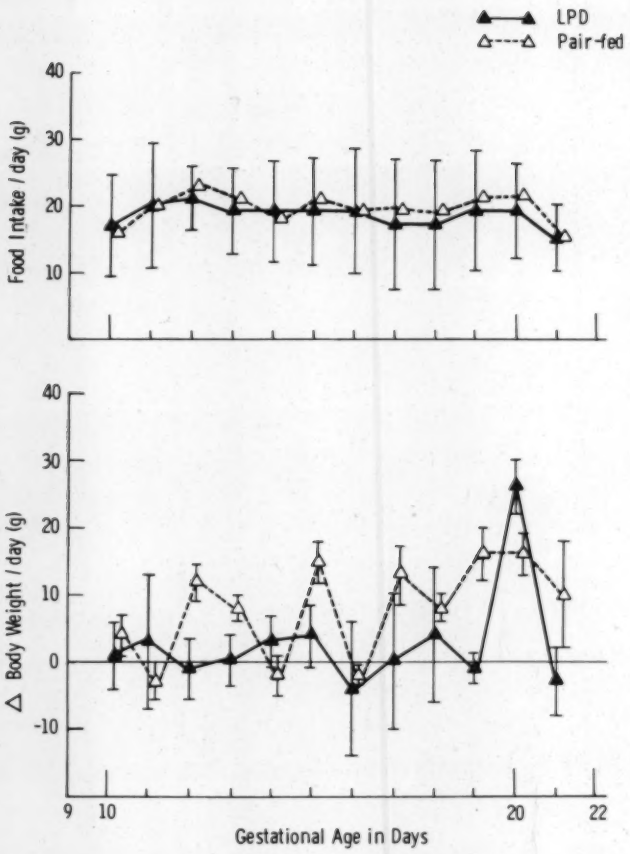

Fig. 3. Food intake and change in body weight of mothers during pregnancy. A 20% liquid protein diet was fed to the mothers between days 9 to 19. From day 19, the diets were changed to a 20% casein diet until birth. Pair-fed mothers received a 20% casein diet throughout the entire experimental period as compared to the 20% LPD group. Pair-fed animals always consumed all the food given.

connective tissue known to be materials of poor protein quality (4). In the present study, the average protein efficiency ratios of the LPD groups was 0.09 compared to 2.12 for the pair-fed groups. Since these hydrolysates from animal sources are deficient in some essential amino acids, the liquid protein products are supplemented with the necessary amino acids (5).

It has been estimated that approximately 100,000 persons have used this diet as a method of weight control (2). As an example, Roberts (7) reported that within a one month period, he has encountered five cases of thromboplebitis in patients resulting from ingestion of a liquid protein diet (LPD). This suggests that the use of LPD for weight control by the general population is more widespread than actually estimated.

The body weights of the offspring from the LPD fed groups were smaller as

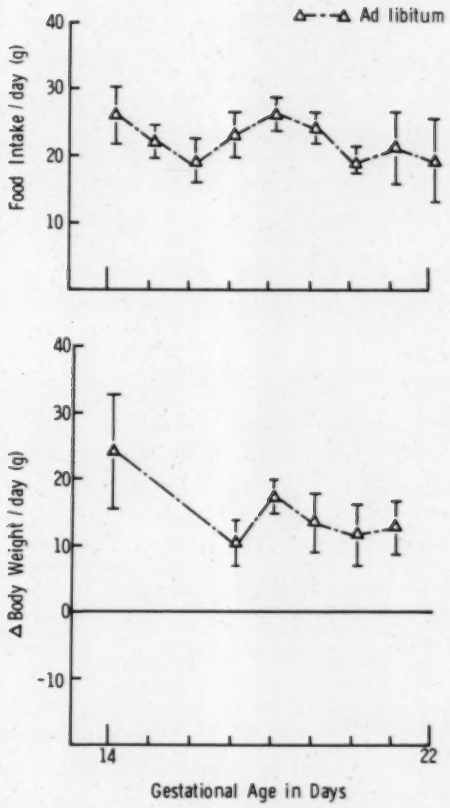

Fig. 4. Food intake and changes in body weight of mothers during pregnancy. Ad libitum groups were fed a 20% casein diet throughout the period. Three mothers of the liquid protein group gave birth at day 21.

compared to the controls. On the other hand, some organs in this group were not necessarily smaller. This may be explained on the basis of the critical period of growth of each organ, as shown in other studies concerned with different nutritional deficiencies (6,8). In each study, body weight stayed the same during the feeding of LPD as compared to the steady increase seen in the pair-fed and ad libitum groups; then the body weights of the group fed LPD showed a sudden large increase within 24 hours upon changing to the casein diet. However, the body weights did not increase continuously with this pace. This unexpected huge increase of body weight in a short time period might be related to the retention of body fluid as a result of increased synthesis of proteins, such as serum albumin (subsequent studies revealed that regardless of the gestational period, this occurs). This suggests that contrary to the description in the "last chance diet" (5), a dieter on liquid protein may experience rapid weight gain when they are released from this dietary regimen.

The animals fed LPD during days 13 to 18 in gestation showed 38% stillbirths, whereas animals fed LPD during 9 to 19 days in gestation only showed 13% stillbirths (10). This apparent discrepancy is explained on the basis of the litter size born to the mothers. In the former group of mothers, an

TABLE 3

Incidences of stillbirths and cataracts

	Stillbirths	Cataracts
LPD fed*	Total Avg. Incidence (30%)	Total Avg. Incidence (15%)
Exp. 1 (13-18) (11)	49/128	9/128
Exp. 2 (9-19) (8)	7/56	18/56
Pair-fed**	Total Avg. Incidence (1%)	Total Avg. Incidence (0%)
Exp. 1 (13-18) (3)	1/38	0/38
Exp. 2 (9-19) (3)	0/37	0/37
Ad libitum***	Total Avg. Incidence (11%)	Total Avg. Incidence (0%)
(13-22) (4)	4/37	0/37

*Liquid protein diets were fed between each gestational age, designated by days. After that, animals were fed a normal 20% casein diet until birth. Figures within the parenthesis show the number of pregnant animals used.

**These animals were pair-fed with the LPD group while they were given liquid protein, as well as when the LPD animals were returned to the 20% casein diet until birth.

***These animals were fed a 20% casein diet ad libitum during the period. The denominator represents the total number of newborn rats born from mothers in each group. The numerator represents the incidence of abnormalities within the total number of newborn rats in each group.

Fig. 5. Cataract formation was present in the newborn whose skin covering the eye had been removed to make this defect more visible. This occurred from feeding a liquid protein diet.

average litter size was 11.6 as compared to 7.0 in the latter, suggesting that some of the litters might have been resorbed before delivery, thus causing the lowering of the stillbirth rate. It has been well documented that protein deprivation during pregnancy increases the rate of stillbirths in rats (10), monkeys (11) and humans (12).

A lack of dietary tryptophan to young growing rats has been known to cause cataracts (13,14). Liquid protein diets contained only about 40% of the recommended requirement (15) of this amino acid during pregnancy. However, additional supplementation of tryptophan did not eliminate the cataract formation (unpublished observation). Some variability exists between those mothers fed LPD whose offspring show cataract formation and those who produce normal newborns. For example, an LPD mother who produced a litter with one newborn exhibiting cataracts is likely to have additional offspring with the same defect. That LPD mother producing normal offspring is likely to have all newborns without the defect.

Subsequent studies at this laboratory have revealed additional defects in newborn rats born to mothers fed LPD until delivery. For example, spinal curvature and dry, wrinkled skin occurred infrequently in only those animals fed LPD (Fig. 6). Craniofacial abnormalities were observed which generally consisted of an underdeveloped maxilla and mandible, as well as a general lack of differentiation, with rounded contours of the cranial region (Fig. 7). This was never exhibited to ad libitum and pair-fed groups. Subcutaneous hemorrahges were found exclusively in those newborns whose mothers received liquid protein during pregnancy (Fig. 8). This suggests a possible defect in the cardiovascular system at the capillary level. There have been clinical reports (7) of thrombophlebitis, in which patients have taken LPD sometime before the onset of the disease. Unexpected deaths of avid users of LPD due to cardiac failure are also well documented (1).

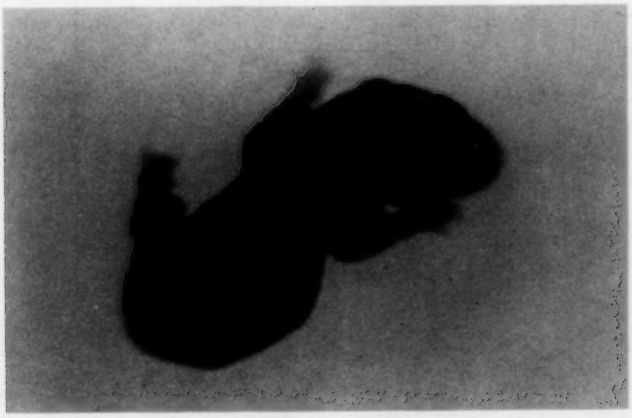

Fig. 6. Spinal curvature and dry, wrinkled skin appeared in this newborn fed a liquid protein diet.

The current data indicate increased risks associated with using liquid protein products during pregnancy. It is important to note that the present dietary regimen was a simple replacement of liquid protein for the casein portion. The total diet contained adequate amounts of carbohydrates, fats, vitamins and minerals for normal health. However, most people using liquid

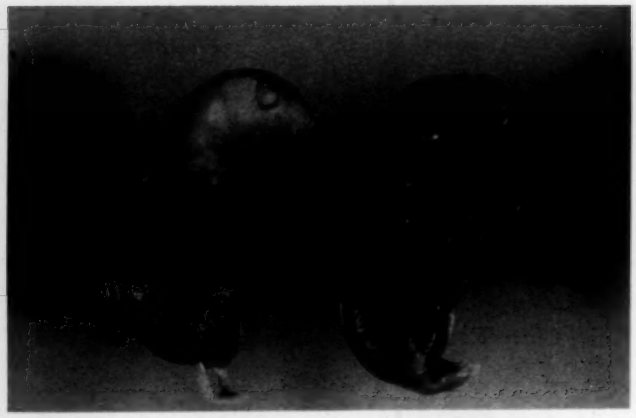

Fig. 7. The unusual cranial features of stillborns fed liquid protein were apparent, showing an underdeveloped maxilla and mandible in the newborns on the left. On the right, an offspring showed normal craniofacial features.

Fig. 8. Subcutaneous hemorrhages appeared in several locations over the surface of the body in newborns fed a liquid protein diet. This appeared similar to ecchymoses.

protein products generally take them as their sole source of nourishment. Thus, the implications of using only liquid protein during pregnancy may be more serious than the present data indicate. The present study was initiated during the middle stage of gestation for the reasons mentioned earlier. It would be interesting to see whether the same abnormalities would result in animal models when this dietary regimen is consumed in the early stages of pregnancy.

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EFFECT OF TYPE AND AMOUNT OF DIETARY FAT ON CONCENTRATION OF CHOLESTEROL IN BLOOD PLASMA AND TISSUES OF RABBITS[1,2]

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ABSTRACT

Rabbits were fed skim milk-based diets containing by weight either 14% soybean oil, 28% soybean oil, 14% tallow or 28% tallow. A control group was fed a commercial stock diet. Plasma cholesterol concentration was monitored for 10 weeks, after which cholesterol concentrations of liver, skeletal muscle, adipose tissue, brain, heart, aorta, kidney, skin and intestinal mucosa were determined. Feeding soybean oil diets (relatively high content of polyunsaturated fat by acids) resulted in lesser concentrations of cholesterol in blood plasma than did feeding tallow diets (greater content of saturated fatty acids). Conversely, cholesterol contents were greater in skeletal muscle, adipose tissue, heart, aorta, kidney, skin and intestinal mucosa of rabbits fed soybean oil diets. Cholesterol contents of liver and brain were unaffected by type of dietary fat. Our results demonstrate that the degree of saturation of fat in a milk-based diet of rabbits will influence the partitioning of cholesterol between the blood and several other tissues and organs.

Key Words: rabbits, soybean oil, beef tallow, tissue cholesterol, body composition.

INTRODUCTION

We previously have examined effects of diets made from skim milk plus either animal or vegetable fats upon growth, health and cholesterol accumulation in the young of several species of animals. Feeding milk-based diets containing soybean oil without added cholesterol to calves, pigs and rats has produced greater cholesterol concentrations in liver, adipose tissue and total carcass than did the feeding of similar diets containing beef tallow (1-5). Others also have observed increased tissue cholesterol in growing lambs, rats and rabbits in response to consumption of polyunsaturated

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[2]A portion of this report was presented at the 65th annual meeting of the Federation of American Societies for Experimental Biology in Atlanta, Georgia, April, 1981 (Fed. Proc. 40:352, 1981).
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fats (6-8). Human infants consuming infant formulae based on milk protein plus vegetable fat grow and develop normally (9), but few data are available regarding cholesterol concentration and distribution in their bodies. To increase understanding of the impact of such milk-based dietary regimes on cholesterol disposition in an animal body, we evaluated the effects of skim milk-based diets containing either soybean oil or beef tallow on the partitioning of cholesterol among various tissues of young, growing rabbits.

MATERIALS AND METHODS

Fifty-two 6-week-old female New Zealand White rabbits were assigned randomly to four dietary groups; eight more were assigned as controls. Average body weights of rabbits in each group ranged from 1305±106 to 1996± 100 grams. Rabbits were fed for 10 weeks one of four fat-supplemented diets based on skim milk (formula 202, low heat Grade A non-fat dry milk, Associated Milk Producers, Inc., San Antonio, TX) or a rabbit stock diet (Teklad, ARS/Sprague Dawley, Winfield, IA) as described in Table 1. Both soybean oil (SBO) (Edsoy, Staley Manufacturing Co., Decatur, IL) and beef tallow (T) were incorporated into diets, hereinafter called fat diets, containing either 14 or 28% fat by weight, which is equivalent to 30 or 50% of total calories, respectively. Protein content was 22% in the 14%-fat diets and 17% in the 28%-fat diets. The two SBO and two T diets were supplemented with coarse-milled stock diet (Table 1) to increase palatability and to promote good health. Each fat diet was formed (by use of a sausage-making die) into pellets that resembled the stock diet in size and appearance. The pellets were dried in a forced-air oven.

TABLE 1
Composition of Diets

Diets	Soybean oil (SBO)	Tallow (T)	Nonfat dry milk	Stock diet[a]	Vitamin-mineral premix[b]
	g/kg				
28% SBO	280	---	514.3	200	5.7
28% T	---	280	514.3	200	5.7
14% SBO	140	---	654.3	200	5.7
14% T	---	140	654.3	200	5.7
Stock	---	---	-----	994.3	5.7

[a]Teklad rabbit diet consisted of ground wheat, soybean meal, pulverized barley, 1% dicalcium phosphate, suncured alfalfa meal and cane molasses. Crude protein=17.0%, crude fat=1.5%, and crude fiber=16.0%.

[b]Premix contained, on an air-dry-weight basis, 0.85% of Rovamix A-300W (300,000 I.U. vitamin A/g), 0.10% of Rovamix AD_3 300/300W (300,000 I.U. each of vitamins A and D_3/g), 70.00% of Rovamix E-100W (100,000 I.U. dl-α-tocopheryl acetate/454g), 24.78% of MgO and 4.27% of chlortetracycline.

Rabbits were provided both food and water ad libitum and were housed in individual stainless-steel cages in a well-ventilated room maintained at 20°C and provided with a 12-hour light cycle. Individual feed intakes were determined daily, and body weights were recorded weekly. Blood samples were collected weekly from a marginal ear vein of each rabbit. Plasma samples prepared from heparinized blood were assayed for total cholesterol content by a colorimetric procedure (10). At week 10, rabbits were killed by air embolism and exsanguinated. The following organs and tissues were removed, weighed and stored at -20°C: liver, gastrocnemius muscle, perirenal adipose tissue, brain, ventricles of heart, aorta, kidney, skin from behind the shoulder and mucosal cells of small intestine. The small intestine was flushed with 0.15 M sodium chloride, opened sagitally and scraped with a razor blade to collect mucosal cells. Preliminary work had shown no difference in

cholesterol concentration in mucosal cells from throughout the length of small intestine. Collected organs and tissues were lyophilized, weighed, ground through a Wiley mill and then sampled. After duplicate 1 g samples each were extracted with 100 ml of isopropanol (2), extracts were analyzed for cholesterol concentration by the same procedure used for plasma (10). Remaining visible adipose tissue, skin and skeletal muscle were dissected from each carcass and weighed to provide an estimate of total tissue mass.

Data were analyzed with a one-way analysis of variance model utilizing contrasts (11).

RESULTS

The growth characteristics of rabbits fed the five different diets are summarized in Table 2. No significant variation among dietary groups in body weight gains appeared during the 10-week period. Although there were death losses early in the experiment, all rabbits that completed the trial were in good health as shown by steady weekly body weight gains, normal amounts of body fat, normal appearance of fur and ordinary behavior. Type of dietary fat (SBO vs. T) did not significantly ($P<0.05$) affect body weight gain. Concentration of dietary fat (14% vs. 28%) also did not affect body weight gain; rabbits merely consumed less of the diets having the greater caloric density.

TABLE 2
Influence of Type and Amount of Dietary Fat on Body Weight Changes and Feed Consumption of Rabbits

Diet	No. of rabbits	Final body weight	Body weight gain[a,b]	Feed consumed[a]	Calculated caloric density of diets
		g			kcal/g
28% SBO	13	3,467±117[c]	1,766±151	5,661±248	5.0
28% T	11	2,981±139	1,681±121	4,857±308	5.0
14% SBO	10	3,529±231	1,735±178	6,682±409	4.2
14% T	9	3,127±119	1,660±102	5,229±168	4.2
Stock	8	3,492±145	1,818±118	11,798±443	ND[d]

[a]Gain in body weight or feed consumed during the 10-week feeding period.

[b]No significant differences between means for different dietary treatments.

[c]All values are means±SEM

[d]Not determined for this diet.

With the exceptions of those for liver and brain, tissues of SBO-fed rabbits had greater concentrations of cholesterol per gram of dry tissue (Table 3). Liver cholesterol concentration, although unaffected by type of dietary fat, was significantly greater in rabbits fed the 28% fat diets than in those fed the 14% fat diets (Figure 1). Cholesterol concentrations in liver and aorta of rabbits fed fat-supplemented diets were greater ($P<0.05$) than those in the same tissues of rabbits fed stock diet, but cholesterol concentrations in other tissues were either not different or lesser in the group fed fat supplemented diets (Table 3).

Influence of amount and type of fat in the diet on cholesterol in an entire organ or tissue was estimated by multiplying cholesterol content of the measured portion by the weight of the whole organ or tissue. Results of these determinations are expressed on a per-kilogram basis to account for differences in sizes of organs and tissues and are presented in Table 4.

TABLE 3
Influence of Type and Amount of Dietary Fat on Tissue Cholesterol Concentrations in Tissues of Rabbits

Diet[a]	Liver	Skeletal muscle	Adipose tissue	Brain	Heart	Aorta	Kidney	Skin	Intest. mucosa
	mg/g dry tissue								
28% SBO	20.3[b] ±3.0	7.1 ±1.4	3.2 ±0.1	78.4 ±1.7	12.0 ±1.4	5.4 ±0.2	16.5 ±0.5	19.3 ±3.5	13.1 ±0.9
28% T	16.3 ±1.8	2.8 ±0.1	1.7 ±0.1	69.2 ±3.2	6.5 ±0.3	3.8 ±0.2	14.1 ±0.7	4.5 ±0.3	7.8 ±0.5
14% SBO	13.5 ±1.7	3.4 ±0.1	3.4 ±0.2	64.0 ±2.6	10.3 ±0.8	5.0 ±0.2	17.4 ±0.6	17.9 ±3.2	12.2 ±0.6
14% T	13.5 ±2.0	2.3 ±0.2	1.8 ±0.1	71.5 ±3.1	5.5 ±0.3	4.5 ±0.3	14.4 ±0.9	4.5 ±0.4	8.9 ±0.7
Stock	9.4 ±0.5	3.3 ±0.3	5.2 ±1.7	79.9 ±1.2	6.9 ±0.3	3.6 ±0.1	16.5 ±0.4	12.7 ±1.3	12.7 ±0.2
Significance[c] (P<0.05)	Amt Stock	Type Amt	Type Stock	Amt Int Stock	Type	Type Int Stock	Type	Type	Type Stock

[a]Number of rabbits fed each diet is indicated in Table 2.

[b]Each value is mean±SEM

[c]Comparisons are SBO vs. T (Type); 14% vs. 28% (Amt); interaction of Type and Amt (Int.); and Fat vs. Stock (Stock)

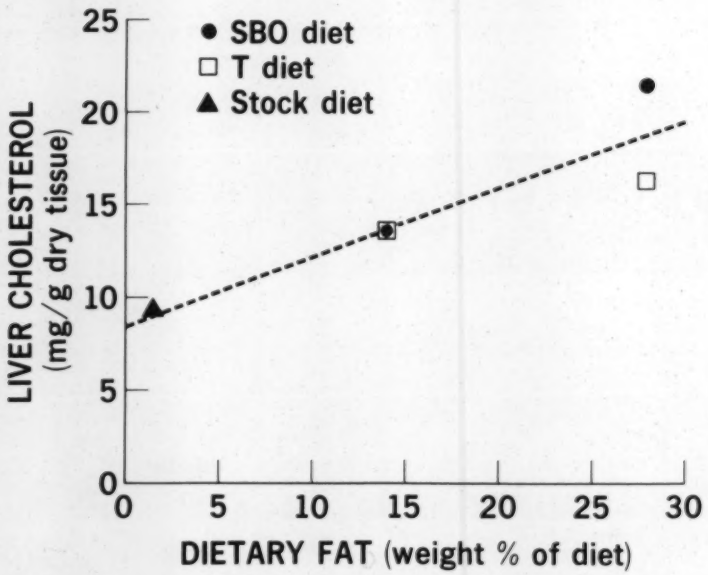

FIG. 1
Concentration of Cholesterol in Liver As a Function of Amount and Type of Dietary Fat.

TABLE 4
Influence of Type and Amount of Dietary Fat on Total Cholesterol Content of Tissues

Diet[a]		Liver	Skeletal muscle	Adipose tissue	Brain	Heart	Aorta	Kidney	Skin	Intestinal mucosa	Total
		mg/kg body weight									
28% SBO		157	3,184	208	43.6	3.0	0.6	17.1	870	33.7	4,518
	%[b]	3.5	70.5	4.6	1.0	0.1	0.0	0.4	19.3	0.7	
28% T		136	971	71.1	38.5	2.0	0.3	15.6	129	23.0	1,386
	%	9.8	70.0	5.1	2.8	0.1	0.0	1.1	9.3	1.7	
14% SBO		112	1,544	214	34.8	3.2	0.4	20.4	730	32.6	2.691
	%	4.1	57.4	8.0	1.3	0.1	0.0	0.8	27.1	1.2	
14% T		104	1,002	57.2	45.4	2.5	0.7	16.4	143	19.4	1,411
	%	7.3	72.4	4.1	3.2	0.2	0.0	1.2	10.1	1.4	
Stock		91.9	1,439	213	46.1	2.3	0.4	18.4	465	25.4	2,302
	%	4.0	62.5	9.3	2.0	0.0	0.0	0.8	20.2	1.1	

[a]Number of rabbits fed each diet is indicated in Table 2.

[b]Percentage of total cholesterol (dry-matter basis) in the tissues measured. Average dry-matter content of skeletal muscle was derived from dry-matter contents of dissected muscles from 5 rabbits per group; dry-matter contents of the other tissues represent the average of those from each respective tissue from all rabbits in each group.

Amount of total cholesterol in each organ and tissue per kg body weight of rabbits in each dietary group ranked from greatest to least as follows: 28% SBO > 14% SBO > stock > 14% T ≅ 28% T. Most of the "additional" body cholesterol in the SBO-fed rabbits was present in skeletal muscle, adipose tissue and skin. Skeletal muscle and skin accounted for more than 80% of the sum of measured cholesterol in each dietary groups.

The weekly cholesterol concentration data of blood plasma were averaged for the 0-2, 3-6, 7-10 and 0-10 week time periods (Table 5).

TABLE 5
Effect of Type and Amount of Dietary Fat on Plasma Cholesterol Concentration of Rabbits

Diet[a]	Time periods[b] 0-2	3-6	7-10	0-10
	mg/dl			
28% SBO	88±10[c]	100±6	119±6	103±4
28% T	103±9	135±9	114±10	119±8
14% SBO	56±5	70±5	88±15	72±7
14% T	112±12	149±18	98±10	120±12
Stock	67±5	51±4	64±7	60±5
Significance[d] (P<0.05)	Type Int Stock	Type Int Stock	Amt Stock	Type Amt Int Stock

[a]Number of rabbits fed each diet is indicated in Table 2.

[b]Time period is weeks of the 10-week feeding period.

[c]Each value is mean±SEM for all values obtained during indicated time period.

[d]Comparisons are SBO vs. T (Type); 14% vs. 28% (Amt); interaction of Type and Amt (Int) and Fat vs. Stock (Stock).

Although analysis of variance of the cholesterol concentration data for the 0-2, 3-6 and 7-10 week time periods did not produce the same statistically significant differences for each time period, the following comments are based on significant differences found for the 0-10 week time period. Rabbits fed T-containing diets had greater concentrations of plasma cholesterol than did those fed SBO-containing diets. Within individual fats, rabbits fed the greater percentage of calories as fat also had greater concentrations of cholesterol in plasma. A statistically significant interaction of type and concentration of dietary fat on plasma cholesterol was observed. Furthermore, rabbits fed the fat-supplemented diets had greater plasma cholesterol concentrations than did rabbits fed the stock diet.

DISCUSSION

A number of workers have reported that the amount of cholesterol circulating in blood and stored in tissues of animals is affected by the source of dietary fat. With the notable exception of the suckling calf (2,4) and the guinea pig (12), most animals and humans exhibit lesser blood cholesterol concentrations when consuming unsaturated fats than when consuming saturated fats. In our experiment, rabbits consuming T, a relatively saturated animal fat, showed increasing plasma cholesterol concentration up to 4-6 weeks on the test diet, after which a gradual decline to the initial value occurred. Connor et al. (13) also observed a short-term hypercholester-

olemic response after feeding a highly saturated vegetable fat (coconut oil) to rabbits.

Liver, an important site for cholesterol synthesis and catabolism, also acts as a reservoir for excess cholesterol (14). In our experiments, the concentration of cholesterol in liver, unlike most other tissues, was influenced by amount of fat in the diet but not by type of fat; consumption of greater amounts of dietary fat increased the cholesterol concentration in rabbit liver. Conner et al. (13) also demonstrated that greater amounts of fat in the diets of rabbits increased cholesterol contents of liver.

Adipose tissue also has been proposed as a site of cholesterol storage (5, 14-17). We found that both SBO and T feeding reduces cholesterol concentration (mg/g of dry tissue, Table 3) in adipose tissue as compared with feeding stock diet. When content of cholesterol in all the dissectible adipose tissue was calculated, rabbits fed the SBO-containing and the stock diets had similar amounts of cholesterol within adipose tissue. Rabbits fed T diets had less dissectible adipose tissue as well as less cholesterol on a per gram basis than did rabbits fed the other diets.

Skin of SBO-fed rabbits contained a comparatively large quantity of cholesterol. Concentrations could have been overestimated because our cholesterol assay did not exclude other sterols having the 5-ene, 3 β-ol configuration. Skin may contain appreciable quantities of noncholesterol animal sterols of endogenous origin as well as from plant products in the diet (18). Refined SBO fed to our rabbits contained negligible quantities of plant sterol according to both the supplier and our own analyses. Negligible sterol content in SBO that we fed and relatively poor absorption of plant sterols (19,20) should have eliminated diet as a significant source of noncholesterol sterols.

In other work (1-5), we have approached the unresolved issue of how unsaturated fats decrease plasma cholesterol by testing the tissue redistribution of cholesterol hypothesis in calves, pigs and rats fed milk diets supplemented with SBO or T. As in the previous studies (1-5), the present study with rabbits has shown greater accumulation of cholesterol in tissues of animals fed SBO than in tissues of those fed T. The observations from this study, unlike some of our earlier studies with other animal species, resemble the human response (21,22) in that the cholesterol concentration of plasma was less when SBO was fed than when T was fed. Other plasma cholesterol-lowering mechanisms in the rabbit, such as decreased rates of absorption or increased rates of excretion, have been investigated and discounted (18). Our work corroborates kinetic data of Hough and Bassett (18) and shows that the amount of cholesterol deposited in tissues depends on at least the type of dietary fat.

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EFFECT OF DIETARY HISTIDINE ON HISTIDINE-CATABOLIZING ENZYMES IN THE CHICKEN (Gallus domesticus)

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ABSTRACT

Experiments were conducted to determine the influence of dietary histidine on histidine catabolism in the chick and to determine possible nutritional interrelationships between histidine and potassium salts of metabolizable organic anions. Supplemental histidine (5.5-6.0%) decreased weight gain and food consumption of chicks, increased liver size, and doubled the activities of liver histidase (EC 4.3.1.3) and histidine-pyruvate aminotransferase (EC 4.2.1.49). Histidine depressed plasma potassium concentration; no other interaction of potassium and histidine was observed.

Key Words: Histidine, enzyme catabolism, growth

INTRODUCTION

Diets that contain a single amino acid at a disproportionately high level are known to be growth-depressing for growing animals. This effect is exacerbated if the basal diet is low or marginally adequate in total protein; much of the growth depression can be alleviated if the total percentage of dietary protein is increased such that there is no longer a relative excess of any one amino acid (1-4). In the case of an excess of the amino acid lysine, the addition of arginine or a high level of sodium or potassium salts of metabolizable organic anions can ameliorate the growth depressing effects of the high lysine diet (5-9). The mechanism by which the salts function is not yet clear, although it is known that the rate of in vivo oxidation of lysine to CO_2 is increased in response to potassium bicarbonate in those animals receiving excess lysine. The activity of the first, and apparently rate-

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limiting, enzyme in lysine degradation, lysine-α-ketoglutarate oxidoreductase, is also increased under these conditions (9).

The enzymes of histidine metabolism are responsive to a variety of dietary changes. Low protein diets severely reduced the activities of histidase (EC 4.3.1.3) and urocanase (EC 4.2.1.49) in fish (10) and young rats (11), whereas fasting and high protein diets had the opposite effect (10,11). The effect of histidine on these enzyme systems is less clear. Shirmer and Harper (11) reported that the addition of 4% histidine to a 12% protein diet which was fed to young rats caused only a minimal increase in histidase and urocanase activity when expressed per gram liver. Total enzyme activity was increased due to the significant increase in liver size caused by excess histidine. These findings are hard to interpret, as total dietary intake and growth were not reported. Sahib and Murti (12), however, found that activities of these histidine-degrading enzymes were increased by force feeding 1000 mg of casein hydrolysate or 50 mg hydrolsate in conjunction with 200 mg of L-histidine.

Histidine-pyruvate aminotransferase activity increased in young rats in response to excess dietary protein (11), but, in contrast to histidase and urocanase activities, also increased in livers of protein-deficient rats (11). In the studies of Sahib and Murti (12) and Morris et al (13), however, dietary protein did not influence histidine-pyruvate aminotransferase activities. Schirmer and Harper (11) reported that 4% L-histidine·HCl added to a 12% casein diet for rats was without effect on hepatic histidine-puruvate aminotransferase activity.

Activities of histidine-metabolizing enzymes in the chick have not been reported previously. The present studies were conducted to evaluate the response of chicks to excess dietary histidine, and to determine the possible interaction of histidine and organic potassium salts.

MATERIALS AND METHODS

Animals

Leghorn cockerels were housed individually or in groups in electrically heated battery brooders with raised wire floors, with controlled lighting cycle of 15 hr light, 9 hr dark. The facility was maintained at 22.5°C, food and water were supplied ad libitum. The animals were maintained on a commercial chick starter until the day prior to initiation of the experiment: they were then fasted overnight and assigned randomly to the treatment groups.

Diets

Experimental diets used in these studies were mixed in our laboratory within two weeks of use. The composition of these diets is shown in Table 1.

Enzyme assays

Histidase: The assay system used to measure histidase (L-histidine ammonia lyase, EC 4.3.1.3) activity was adapted from Spolter and Baldridge (14). Livers were removed from decapitated birds and minced in ice cold 1% KCl, and homogenized in 1% KCl. A 0.1 ml aliquot of 10% homogenate was preincubated with 0.2 ml of 0.1 sodium pyrophosphate buffer (pH 8.8) for 10 minutes at 37°C in a shaking water bath. The reaction was initiated by the addition of 0.1 ml of histidine (200 µm) and terminated by the addition of 0.5 ml of 5% perchloric acid. Blanks were identical in composition, with the order of addition of the histidine and perchloric acid reversed. After termination, the reaction tubes were centrifuged and the supernatant removed. The amount of

TABLE 1

Composition of Diets

Ingredient	Diet A (%)	Diet B (%)
Soy protein		26.33
Corn gluten	33.34	
Corn oil	3.50	4.00
Cellulose	6.00	6.00
DL-methionine		0.60
Arginine HCl	0.58	
L-tryptophan	0.20	
Lysine HCl	0.79	
Glycine		0.40
Vitamin mix[a]	1.22	1.22
Mineral mix[b]	5.83	5.83
Trace mineral mix[c]	0.10	0.10
Glucose monohydrate	48.44	55.52

[a] Supplied the following as mg/kg of diet: thiamin·HCl, 15.0, riboflavin, 15.0; nicotinic acid, 50.0; d-calcium pantothenate, 20; pyridozine·HCl, 6.0; folic acid, 6.0; biotin, 0.6; inositol, 250.0; menadione sodium bisulfite, 1.52; butylated hydroxytoluene, 100.0; choline chloride (70% solution), 2200.0; glucose monohydrate, 7020.48. Also supplied in IU/kg of diet: Vitamin D_3, 4500; Vitamin A palmitate, 5000; Vitamin E acetate, 110.

[b] Composition of mineral mix (%); $CaHPO_4 \cdot 2H_2O$, 42.88; $CaCO_3$, 17.91; $NaHCO_3$, 12.73; naCl, 4.24; $KHCO_3$, 17.10; $MgSO_4 \cdot 7H_2O$, 5.14.

[c] Composition of trace mineral mix (%): $MnSO_4 \cdot H_2O$, 34.95; KIO_3, 1.00; $FeSO_4 \cdot 7H_2O$, 49.95; $CuSO_4 \cdot 5H_2O$, 3.00; ZnO, 10.00. $CoCl_2 \cdot 6H_2O$, 1.00, $NaMoO_4 \cdot 2H_2O$, 0.83; Na_2SeO_4, 0.10.

urocanic acid formed was determined by comparison with the standard curve of urocanate measured spectrophotometrically at 227 m μ. Preliminary experiments demonstrated a pH optima in chickens of 8.6. The reaction was not linear after 10 min; thus, 10 min incubations were used in these studies.

Histidine-pyruvate aminotransferase: The basic outline of the histidine-puruvate aminotransferase assay was provided by Spolter and Baldridge (14). Aliquots of minced livers were homogenized in 0.14 M KCl (1/10) containing .005 normal NaOH. The assay consisted of preincubating 0.1 ml pyridoxal phosphate (0.3 mg per ml), 0.8 of 0.1 M EDTA, 1.4 ml borate arsenate buffer[3] and 0.2 ml of 0.8 M sodium pyrophosphate with 0.5 ml of homogenate for 5 minutes at 37°C. The reaction was initiated by the addition of 0.5 ml of 0.3 M L-histidine.

[3] 1.43 M sodium borate, 0.5 M sodium arsenate, pH 8.5.

Following a 20-min incubation at 37°C, the reaction was terminated with the addition of 1.0 ml 10% perchloric acid. The precipitated protein was removed by centrifugation and the supernatant used for determination of imidazolepyruvic acid. Preliminary experiments demonstrated that the rate of imidazolepyruvic acid formation decreased after 20 min, which was therefore used as the standard incubation duration. The pH optima of histidine-pyruvate aminotransferase in the chick was determined to be 8.5. Activities obtained using these two assay systems were similar to those reported in young rats (11).

Blood pH was determined using a blood gas and pH analyzer. Plasma potassium analyses were made using an atomic absorption spectrophotometer. Heparinized plasma was diluted 1:50 with 0.2 normal $LiNO_3$ prior to assay, lithium serving as an ionization supplement. Plasma histidine was determined in protein-free supernatant using an amino acid analyzer.[4]

All data were subjected to analysis of variance (15). When significant differences among treatments were indicated by this procedure, the Duncan's multiple range test (16) was used to determine differences between treatments.

TABLE 2

Physiologic Effects of Histidine and Potassium, Experiment 1

Supplement to basal diet[a]	Weight gain[b,c] (g/chick)	Blood pH[c,d]	Liver size[c,e] (% B.W.)	Plasma histidine[c,d] (mm/ml)
None	176.4±4.6^{A}	7.41±.03^{A}	3.19±.04^{A}	.14±.01^{A}
Histidine[f]	106.9±4.1^{B}	7.43±0.3^{A}	5.79±.72^{B}	4.23±.72^{B}
Potassium	169.4±4.1^{A}	7.45±.04^{A}	3.05±.09^{A}	.13±.00^{A}
Histidine & Potassium[g]	113.5±3.8^{B}	7.50±0.2^{A}	4.25±.42^{B}	3.70±.37^{B}

[a] Diet B, Table 1. Chicks were fed the diet from day 11 to day 28 posthatch.

[b] Mean ± SEM of 10 chicks.

[c] Means within columns having the same capital letter in their superscripts are not significantly different (P>0.05).

[d] Means ± SEM of 4 chicks, drawn randomly from each experimental group.

[e] Mean ± SEM triplicate pool of 2 chicks each.

[f] 6% histidine (free base) was added at the expense of glucose monohydrate.

[g] 1.4% potassium was added as potassium acetate at the expense of cellulose.

[4] Technicon TSM amino acid analyzer, Technicon Instruments Corporation, Tarrytown, NY

Experiment 1

The effects of excess dietary histidine and potassium acetate on chicks are shown in Table 2. The addition of 6% histidine to the basal diet resulted in a marked growth depression and increase in liver weight. Plasma histidine concentration increased 30 fold. When potassium acetate alone was added to the basal diet, there was no apparent effect on growth, liver weight, or histidine status as estimated by plasma concentration. When the two were fed simultaneously, growth was not improved, nor was the magnitude of liver enlargement significantly affected.

Experiment 2

The results of Experiment 2, presented in Table 3, confirm the previous finding that potassium salts or organic acids did not alleviate the growth depression of chicks fed a diet containing a large excess of histidine.

TABLE 3

Physiologic Effects of Histidine and Potassium, Experiment 2

Supplement to basal diet	Weight gain[b,c] (g/chick)	Feed efficiency[c,d]	Liver size[c,e] (% B.W.)	Plasma potassium[c,f] (mEq/liter)
None	136.5±3.8^{A}	.701±.013^{A}	3.14±.08^{A}	7.84±.37^{AB}
Histidine[g]	93.3±2.2^{B}	.466±.008^{B}	4.75±.20^{B}	6.13±.05^{C}
Potassium[h]	133.4±2.2^{A}	.690±.004^{A}	3.20±.11^{A}	7.94±.06^{A}
Histidine & Potassium[g,h]	82.2±2.7^{B}	.427±.008^{B}	4.00±.22^{B}	6.93±.18^{B}

[a] Diet A, Table 1. Chicks were fed one experimental diet from day 10 to day 25 posthatch.

[b] Each value represents mean ± SEM of triplicate pens of 6 chicks each.

[c] Means within columns having the same capital letter in their superscripts are not significantly different ($P<0.05$).

[d] Grams live weight gained per chick ÷ grams food consumed per chick.

[e] Each value is the mean ± SEM of triplicate pools of 2 chicks each, drawn randomly from each group.

[f] Each value is the mean ± SEM of triplicate pools of equal volumes of plasma from 2 chicks.

[g] Histidine (free base) (5.5%) added at the expense of glucose.

[h] Potassium (1.4%) added as potassium carbonate at the expense of cellulose.

Potassium similarly had no effect on the efficiency of feed utilization, which was predictably low in the birds receiving excess histidine. Adding histidine to the basal diet resulted in a reduction in plasma potassium concentrations of at least 1 mEq/liter; the addition of over 1% potassium as potassium carbonate to the diets containing excess histidine did not increase plasma potassium concentrations.

Table 4 shows the effect of diet on the first enzymes of two pathways of histidine catabolism. Excess histidine resulted in a twofold increase in the activities of both histidase and histidine-pyruvate aminotransferase. Potassium carbonate alone or in combination with histidine, did not affect either enzyme.

TABLE 4

Effect of Histidine and Potassium on Histidine-Catabolizing Enzymes, Experiment 2

Supplement to basal diet[a]	Histidase[b] Urocanic acid formed		Histidine-Pyruvate Aminotransferase[b] Imidazolepyruvic acid formed	
	µm/g liver/hr	µm/mg protein/hr	µm/g liver/hr	µm/mg protein/hr
None	7.56±.26^{A}	2.38±.04^{A}	1.75±.01^{A}	.55±.01^{A}
Histidine[c]	15.68±.75^{B}	4.58±.07^{B}	4.38±.40^{B}	1.28±.06^{B}
Potassium[d]	6.68±.76^{A}	.198±.02^{A}	1.80±.39^{A}	.53±.09^{A}
Histidine & Potassium	16.41±.07^{B}	4.83±.13^{B}	3.98±.16^{B}	1.17±.12^{B}

[a] Diet A, Table 1. Chicks were fed one experimental diet from day 10 to day 25 posthatch.

[b] Each value represents mean ± SEM of triplicate pools of 2 chicks from each group. Means within columns having the same superscript are not significantly different (P>.05).

[c] Histidine (free base; 5.5%) added at the expense of glucose.

[d] Potassium (1.4%) added as potassium carbonate at the expense of cellulose.

DISCUSSION

These studies demonstrate that a large excess of dietary histidine fed to chicks causes growth depression and liver enlargement similar to that found with the young growing rat. In these experiments, the activities of the first enzymes of the two main pathways of histidine metabolism were more than double the control values when histidine was present in excess in an otherwise nutritionally adequate diet. This in contrast to rats in which histidiase is reported to be slightly affected (11,12), and histidine-pyruvate aminotrans-

ferase unaffected (11) by excess dietary histidine (11,12). Chick histidase activity was generally higher than histidine-pyruvate aminotransferase activity in agreement with rat studies (11). It is not clear whether the differences in the chick and rat reflect true species differences in histidine metabolism, or differences in experimental protocol.

Histidine, fed at 5.5 or 6% excess in the diet, caused a significant reduction in plasma potassium on the 15th day of the experiment. These experiments were part of a larger study that included excesses of other amino acids. Lysine, arginine, leucine and threonine also decreased plasma potassium levels (data not shown). This may be the result of an exchange phenomenon similar to that seen during acute administration of other amino acids in man and dogs (17-19); that is, these amino acids may have displaced intracellular potassium and enhanced the urinary excretion of potassium.

Potassium salts did not influence any of the parameters measured in these experiments, with the exception of plasma potassium concentrations in chicks fed excess histidine, lysine and threonine, which were increased by potassium ($P<0.05$). They also did not improve the growth of chicks receiving 3.0% supplemental lysine·HCl in experiment 1 and 1.8% supplemental lysine·HCl in experiment 2 (data not shown). This conflicts with previous findings on lysine-electrolyte interrelationships (5-9) and precludes firm conclusions about nutritional interaction, or lack thereof, between potassium salts and histidine.

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DIETARY PROTEIN AND CALCIUM LOSS; A REVIEW

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ABSTRACT

For several decades, high levels of dietary protein have been suspected to induce hypercalciuria. This increase has been found to be independent of the protein source. Intestinal calcium absorption appears to be increased by the high protein levels; serum calcium levels are not affected. Researchers have found varying effects on calcium balance; some have found that calcium balance is decreased, some have found no difference. Renal handling of calcium does appear to be altered; there is both a decreased renal tubular fractional reabsorption of calcium and an increased glomerular filtration rate. There are some data to suggest that these alterations may be due to the levels of sulfur-containing amino acids in diet. There are other data indicating that changing insulin levels may produce the hypercalciuria. Animal research suggests that there may be an increase in small intestine mucosal surface area.

KEY WORDS: calcium, hypercalciuria, protein, amino acids

INTRODUCTION

Increased dietary protein levels produce hypercalciuria in humans. The mechanism for the hypercalciuria has been under study: it may be due to either an increased excretion of body calcium stores, or to a greater influx of calcium from the gastrointestinal tract, which is then excreted in the urine. Renal alterations in calcium handling appear to be involved in producing the hypercalciuria. Research has been conducted to determine whether all protein-containing foods produce these shifts, or whether they are caused by some specific class of amino acids. Some of the changes resulting in the hypercalciuria may be explained by hormonal shifts which occur during high-protein feeding. Additionally, animal work suggests that there may be morphological changes in the gastrointestinal tract which could explain part of the changes.

HUMAN RESEARCH

Since 1930, diets with high meat content have been known to cause hypercalciuria and negative calcium balance in man (1). The possibility that this was due to an increased acid content in the diet was explored by Farquharson, in 1931 (2), who demonstrated that an increased dietary acid

content did result in increased calcium excretion. In 1939, Kunerth and Pittman confirmed the earlier work and demonstrated that after a three-fold increase in dietary meat content, calcium absorption was increased, yet a negative calcium balance was still produced (3,4). Protein sources other than meat were tested shortly later, and it was demonstrated that increasing the dietary protein content with protein hydrolysate, egg white, or gelatin caused increased calcium absorption (5,6), and that the protein hydrolysate caused hypercalciuria (6,7).

More recent research has confirmed these earlier results. Numerous American researchers have demonstrated recently that increasing the quantity of meat in a diet results in hypercalciuria (8-17). These studies have been performed in both adult men and women (10). The relationship between the dietary protein and the urinary calcium appears to be approximately linear, where an increase in protein from 47 g/day to 95 g/day to 142 g/day resulted in an increase in urinary calcium from 168 mg/day to 240 mg/day to 301 mg/day in one study (14); an increase in protein from 48 g/day to 141 g/day resulted in an increase in urinary calcium from 175 mg/day to 338 mg/day in another study (10); and an increase in protein from 50 g/day to 150 g/day resulted in a doubling of urinary calcium in a third study (16). The same results have been found when purified amino acids were used, where increasing the diet from a low of 0 g nitrogen/day to 90 g N/day resulted in an eight-fold increase in urinary calcium (18). Protein-containing food sources other than meat also produced hypercalciuria. Some of the other foods demonstrated to have this ability include egg albumin, soy protein, casein, lactalbumin, cottage cheese, wheat gluten, and gelatin (18-20). Dietary calcium contents does not appear to affect the hypercalciuric action of dietary protein (11).

There are two different alternatives that these results suggest. The first is that given an increased dietary protein load, the body absorbs more calcium from the gastrointestinal tract. The increased levels of calcium present in the body are then excreted in the urine. The hypercalciuria represents an increased flux of calcium from the gastrointestinal tract to the kidneys in this alternative. This alternative is consistent with either a zero or a positive calcium balance. The second alternative suggests that the increased urinary calcium, rather than coming from an increased gastrointestinal absorption, represents a depletion of body calcium stores. This alternative is consistent with a negative calcium balance.

Measurements have been made of the changes in fecal calcium with dietary protein level, but they are not consistent. Linkswiler reported that increasing the dietary protein with mixed food sources resulted in decreased fecal calcium (12), but later reported no effect (14). It is thus difficult to determine, based solely on measurements of fecal calcium content, whether there is merely increased calcium absorption and decreased calcium excretion from the gastrointestinal tract, or whether there is a loss of body calcium stores.

Studies of intestinal calcium absorption as affected by dietary protein levels have usually demonstrated that, in man, increased dietary protein results in increased intestinal calcium absorption; that is, a decrease in the ratio of fecal calcium to dietary calcium (6,11,19). These studies have included numerous food sources, including mixed foods, casein/lactalbumin, wheat gluten, gelatin, and protein hydrolysates. Some studies, performed for sixty days, have demonstrated no change in the calcium absorption, however (17). Additionally, one study of six individuals at protein levels of 0.9, 12, and 24 g N/day, showed both the highest and the lowest calcium absorptions in the individuals receiving the lowest dietary protein (21). Between the other two groups, though, the individuals receiving the higher dietary protein

content had the higher average intestinal calcium absorption. It is possible, then, that in certain specific circumstances, there is an increased gastrointestinal calcium absorption, which may represent part of the increase in urinary calcium excretion. This does not rule out that body calcium stores may also be depleted.

Serum calcium levels appear to be independent of the dietary protein levels (13,20). Similarly, dermal calcium losses appear to be independent of dietary protein levels (8, 22). Hence, if there is any shift in calcium fluxes, whether from increased gastrointestinal input or from loss of bone stores, the calcium is not being sequestered in the serum compartment, nor is it being excreted in the sweat. The overall changes in calcium balance can thus be determined by measuring solely calcium input, and fecal and urinary calcium excretion.

Studies of the overall change in calcium balance as affected by dietary protein have generally shown that increasing the dietary protein level results in an increasingly negative calcium balance (14,15,17,23). These studies have been performed with both mixed foods serving as the protein source, and with parenterally-administered amino acid mixtures. Cummings found no alteration in overall calcium balance, although there was hypercalciuria, when meat was the protein source (9). Chu found that subjects receiving 12 g N/day had a less negative calcium balance than subjects receiving 24 g N/day, but the subjects receiving 12 g N/day also had a less negative calcium balance than subjects receiving the even lower dietary level of 0.9 g N/day (8). Studying these subjects for radiocalcium kinetics, the calcium turnover was also lowest in subjects receiving 12 g N/day, but was highest in subjects receiving 0.9 g N/day (21). The same is also true for the bone calcium turnover; that is, the subjects receiving 12 g N/day had the lowest amounts of calcium both entering and leaving their bone, as measured by radiocalcium techniques, whereas the subjects receiving 0.9 g N/day had the highest bone calcium turnover (21). Schuette found that increasing dietary protein resulted in increased urinary hydroxyproline excretion, implying a loss of bone matrix and bone calcium (24). These balance data are not sufficient to determine whether there is an increased flux of calcium in from the gastrointestinal tract and out the kidneys, or whether there is a loss of body calcium stores. The hydroxyproline data do imply a loss of body calcium stores.

Spencer, in contrast to other researchers, found no hypercalciuric effect of dietary protein (25). She speculates that this may occur as she is using meat as a protein source, and the increased phosphorus content of the meat may have a protective effect, compared to the more purified diets used in other recent studies. Hegsted did find a hypercalciuric effect of dietary protein, but she also demonstrated that increasing the phosphorus level of the diet reduced the magnitude of the effect (15).

Most American investigators feel that increasing the dietary protein content alters the renal handling of calcium. Several have demonstrated that increasing the dietary protein content results in an increased glomerular filtration rate (GFR) (13,16,17,26), although at least one investigator has not found this change (20). These scientists, among others, have demonstrated a decreased renal tubular fractional reabsorption of calcium with an increased dietary protein content (13,16,17,20,26,27). Schuette demonstrated an increased renal acid and ammonium excretion along with these renal changes (26). These changes, although they do explain the mechanism of the hypercalciuria, do not specify from where the excess excreted calcium is coming.

Several investigators suspect that these renal changes may be due to the role of the sulfur-containing amino acids, which increase the urinary acid load. Lemann and Relman demonstrated that adding methionine to soy flour protein resulted in increased levels of urinary calcium (28). Walser and Browder showed that by infusing dogs with a 0.1 M sulfate solution at 10 ml/min for over one hour, the renal calcium clearance, which is normally about 0.4% of the glomerular filtration rate, is increased to 13-16% of the GFR (29). Zemel fed subjects a diet with either 50 g/day of protein, 150 g/day of protein, or 50 g/day of protein plus additional sulfur-containing amino acids (SAA) to equal the SAA-content of the 150 g/day protein diet (16). He demonstrated that on the 150 g protein diet, there was a doubling of the urinary calcium levels, an increase in the glomerular filtration rate, and a decrease in renal tubular calcium resorption. In subjects on the diet containing 50 g protein plus SAA, he demonstrated changes in the same direction, but of only about half the magnitude as the subjects on the 150 g/day protein diet. This raises the possibility that, although the SAA may play a role in the hypercalciuria, they may not be totally responsible for the increase, and other components of a high-protein diet may play some role. In contrast, Block fed subjects diets consisting of 15 g/day protein, 45 g/day protein, and 15 g/day protein plus SAA (27), and found no difference in urinary calcium levels between those subjects fed the diet with 15 g/day protein, or 15 g/day protein plus SAA. Schuette confirmed the hypothesis recently when she measured changes in urinary calcium that resulted from increasing either dietary protein content or dietary SAA content. She found that although both increased urinary calcium levels, the SAA-containing diet was not as potent as the diet with the increased protein from balanced protein sources (24). She did note that the increase in urinary calcium that resulted from an increased dietary acid load depended upon how the acid was excreted; foods which cause an increase in dietary ammonium, such as proteins and SAA's, are calciuretic; foods which cause an increase in titratable acids (free organic acids and organic acids buffered by phosphate), were not calciuretic.

Other mechanisms for initiating the observed changes have been tested. Increased dietary protein does not appear to affect levels of circulating parathyroid hormone (13,26,30). Similarly, it does not appear to affect circulating levels of 1,25-dihydroxycholecalciferol (26). Recent work indicates that insulin may be affected, however. In subjects fed diets with either 15 g/day or 45 g/day of liquid protein, insulin changes occurred approximately one-half hour before the observed changes in urinary calcium levels and in renal tubular reabsorption, and the changes in the insulin levels appeared to be linearly related to the calcium changes (30). More research needs to be done to confirm this theory.

ANIMAL STUDIES

Rat studies have shown some similarities but some differences from human studies. Increasing dietary protein levels do produce hypercalciuria in the rat (3,8,31,32). There tends to be a marked drop in rat fecal calcium under these conditions, (32,33). This is coupled with an increased intestinal calcium absorption (32,34). Indeed, one study demonstrated an increasingly-positive calcium balance in rats with increased dietary protein (33). These results indicate that the hypercalciuria in the rat results from an increased net gastrointestinal absorption of calcium, and a resultant increased renal excretion, to maintain steady body calcium levels. In those studies which examined bone status, animals fed increased levels of dietary protein did have widening of their epiphyseal plates (35), but other studies showed no change in the bone wet weight, ash weight, calcium content, specific

gravity, or length (34). No body stores of calcium seem to be depleted in the rat, despite the hypercalciuria, confirming that the increased urinary excretion of calcium is merely a shift in fluxes. Radiocalcium studies tested the effects of specific amino acids on the uptake of calcium in bones of rats (36). These studies showed that following a challenge with the basic amino acids lysine or arginine, there was increased appearance of new calcium in bone over that which followed a challenge with tryptophan, leucine, or aspartate. Challenge with all five of these amino acids, however, produced increased appearance of new calcium in bone than after challenge with other amino acids. Other studies of the mechanism of the hypercalciuric effect in rats have shown, by using different protein sources, that the increase in urinary calcium is proportional to the sulfur-containing amino acid levels in the diet (37). The proteins tested included casein, lactalbumen, egg white, casein, and gelatin. Additionally, in the same studies, supplementing the casein with the sulfur-containing amino acids methionine and cystine also resulted in an increase in the urinary calcium, proportional to the SAA content. In addition, the calcium excretion was proportional to the sulfate excretion. Thus, in rats, the hypercalciuric response to increased dietary protein loads appears to be dependent upon the particular amino acids.

One study on the mechanism of calcium absorption in rats fed a high-protein diet demonstrated that in the high-protein animals, the mucosal dry weight per centimeter of length in the duodenum and the ileum was increased in animals fed a 39% casein diet, compared to those fed a 0% or 89% diet. However, the calcium absorption per gram of mucosa was the same in all three groups (38). This study raises the possibility that there is an increased gastrointestinal calcium absorption whose mechanism is by increasing the mucosal surface area, rather than by a membrane effect.

SUMMARY

Increased dietary protein levels produce hypercalciuria. This hypercalciuria results when the protein source is meat, dairy protein, vegetable protein, or purified amino acids. The hypercalciuria appears to be independent of dietary calcium levels. There are two possible explanations for the hypercalciuria: there may either be an increased flux of calcium in from the intestine, which is then excreted through the kidneys; or there may be an actual loss of body calcium stores. Intestinal calcium absorption generally is increased, although overall intestinal calcium flux, which is also affected by endogenous calcium secretion, varies. Serum calcium levels do not appear to be affected by the dietary protein level. Many researchers have found an overall negative calcium balance resulted from a high-protein diet, although some investigators, using diets with a high phosphorus content, found no deleterious shifts in calcium balance. Based on these data, it is not possible to conclusively determine whether there is solely an increased calcium flux from the intestines to the kidneys, an actual loss of body calcium stores, or, as is most likely, a combination of both effects.

Renal handling of calcium is altered in subjects fed a high-protein diet. The glomerular filtration rate is increased. More importantly, the renal tubular fractional reabsorption of calcium is decreased. In addition, renal acid excretion may also be increased.

There do not appear to be changes in the circulating levels of the major calcium regulatory hormones, parathyroid hormone and 1,25-dihyroxycholecalciferol. Changes in insulin levels, however, precede the changes in renal calcium handling.

Some work has suggested that the sulfur-containing amino acids may be responsible for the alterations observed. Research with isolated sulfur-containing amino acids, though, has succeeded in producing changes of only one-half the magnitude of what is observed using diets with balanced amino acid content.

Results of animal research have generally been consistent with the research done in humans. Some rodent studies have suggested, though, that the gastrointestinal changes may be due to increased mucosal surface area in the small intestine, which results in increased gastrointestinal calcium absorption.

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CARNITINE IN THE PERINATAL PERIOD OF MAMMALS

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ABSTRACT

Our present day knowledge on the role of carnitine may be summarized as follows: (1) carnitine is essential for fatty acid oxidation and ketone formation, (2) carnitine is essential for heat production in brown adipose tissue of suckling rats (see also 42), (3) the newborn rat and probably man require carnitine in their food since they cannot make it in sufficient amounts. This is probably not the case in the adult, since surgical patients fed intravenously maintain normal plasma levels of carnitine for at least 20 days

Key Words: carnitine, carnitine acyltransferases, development, parenteral nutrition.

One of the metabolic changes that occur very soon after birth is an increase in the rate of fatty acid oxidation (for review see 1). This takes place to a large extent in the mitochondria. Transfer of the acylCoA into these cell particles requires L-carnitine and carnitine acyltransferases. Interest in this seemingly esoteric substance has increased of late, particularly, since several inborn errors of carnitine metabolism have been reported (for review see 2, 3, 4 5). In the suckling period one tissue in particular requires and contains large amounts of carnitine - brown adipose tissue (6,7). In the blood (plasma) carnitine occurs as free carnitine and as acetyl and acyl (long chain) carnitine, the content of the latter being rather low under normal circumstances (8). The ratio of acyl-carnitine to free carnitine and the absolute level of acetylcarnitine are both positively corelated with the levels of FFA and β-hydroxybutyrate (8,9) and usually indicate the degree of FA oxidation.

In the rat fetus plasma (10) and tissue (see 5,11) levels of both free and acetylcarnitine are low. They rise rapidly after birth together with the acetyl to free carnitine ratio and the blood levels of FFA and ketones (10).

Carnitine content of rat liver rises to very high values 2 days after birth and then falls again, while in heart there is a steady rise (11). Milk carnitine content is about 300 μmoles/l perinatally and falls to below 100 on day 15 after delivery (12).

fatty acid oxidation in brown fat plays a special role in heat production of cold adapted rats (32).

The postnatal rise of hepatic carnitine acyltransferase in rabbits is not dependent on the fat content of the diet. The rise occurs also if a fat free diet is fed (33). The CAT activity for C_8 to C_{12} acids was much higher than that for C_{16} acids (34). This is in good agreement with other work showing that shorter chain FA are preferentially used as a source of energy (35).

Another interesting and provocative finding is the 10 fold lower CAT activity in livers of rats of all ages than in livers of guinea-pigs, rabbits, monkeys and men (27,31,36). The need for carnitine in the neonatal period has also been well documented by Robles-Valdes et al (12) for the rat. They demonstrated milk total carnitine content was highest perinatally (>300 µmoles/ml) and decreased to <100 µmoles on day 16, while plasma levels rose to a maximum on day 5. In liver levels rose about 4 fold perinatally and decreased below fetal values after weaning while a gradual rise up to day 16 was noted in the heart. They injected the mother rat with γ-butyrobetain (methyl-^{14}C) and found C^{14} carnitine only in the rat pups indicating synthesis in the mother only. Thus in neonatal rat pups carnitine is mainly derived from maternal milk. This is underlined by the inability of the newborn liver to synthetize carnitine (26). One interesting mechanism that preserves carnitine (and other substances) is the recirculation between mother and pup: Maternal milk → pup plasma → pup urine → maternal gut → maternal plasma → milk (7). This is due to the fact that neonatal rats cannot micturate spontaneously. The mother evokes micturation by licking the peritoneum and drinking the urine.

This mechanism is, of course not available in man, in whom plasma and urine levels of acetylcarnitine rise sharply after birth (37). Since total carnitine in any fluid (plasma, ammoitic fluid, urine) consists of free and esterified carnitine and since the more carnitine is present as acyl-mostly acetyl-) carnitine the less will be free, any data presenting levels of free carnitine only are meaningless. Thus the most recent reports (38) on the development of free carnitine levels in plasma and urine of newborns and children, suggesting the plasma levels attain adult values by 6 months after birth may equally well mean that at that time less acetyl carnitine is present, because less fat is being oxidized. Similarly, the urinary excretion of carnitine, even in the adult is nearly equally distributed between free and esterified carnitine (e.g. Hahn and Wittmann, submitted for publication) and hence the low urinary excretion of free carnitine up to the 3rd postnatal year suggests a higher excretion of acyl-carnitine. However, this will have to be demonstrated.

Recently Yeh (39) showed that orally administered DL - carnitine (200 mg/100g) decreased blood ketone levels by nearly 40% within 3 hours in 6-day-old rats and in adult starved animals. The author concludes that high carnitine doses suppress hepatic ketone production, since in vitro a 40 mM concentration has an inhibitory effect while 20 mM increase production fourfold. Hence one would expect lower carnitine doses to raise blood levels of ketones, but this did not occur.

1400 mg carnitine/day given for 30 days to infants aged 3 to 6 years lowered blood cholesterol levels from 182 ± 5 to 160 ± 4 mg/dl, but had no effect on TG levels (40). "Lipoprotein" levels were also lowered.

In this review, we have centered attention on the perinatal period. Two recently published reviews should be noted. One (5) also dealing with the neonate, the other considering the need for carnitine in total parenteral nutrition (41).

Finally, for the sake of completeness, we must mention the well-documented but poorly understood role of carnitine in sperm motility (43). From the practical point of view, it should be emphasized that oral and intravenous doses of L-

In rabbits the aortal content of total and acid soluble carnitine increased 6 to 7 fold between week one and 24 after birth. This content was raised 3 to 5 fold if a high fat diet containing cholesterol was fed (13). Weaning to the Purina Chow diet results in a fall in the acetylcarnitine level only. This can be prevented by feeding a high fat diet, particularly if medium chain fatty acids are used (10). The rise in blood acetylcarnitine levels at birth is also found in other mammalian species (14,15), including man (16). In newborn infants fed a soybean formula diet or maintained solely on I.V. nutrition with or without intralipid, blood levels of total carnitine decrease (17,18,19,21) together with the rate of urinary excretion and tissue carnitine content (20). Similarly if newborn rats are not fed at birth, their blood level of total carnitine is not increased 16 hours after birth as it is in normally suckled pups (unpublished data). This suggests that carnitine must be supplied in the food. Breast milk contains 39-63 nmoles/l of L-carnitine while no carnitine was found in soybean formula or intralipid (21). A detailed analysis of the carnitine content of infant foods has been published (22). The perinatal rise in carnitine blood levels in the rat and sheep, the rise in cardiac and muscular carnitine content in man (5), and the fall in infants plasma fed diets not containing carnitine suggest that these newborns are not capable of producing sufficient carnitine themselves. This is supported by the finding that the urinary excretion of carnitine is also considerably diminished in newborns fed soy bean formulas (17).

Carnitine is synthetized from lysine and methionine. Its precursor γ-butyrobetain can be formed in several tissues. However, the last step catalyzed by a hydroxylase occurs only in the liver in rats (23,24), and predominantly in the kidney in man (25). There are only 2 reports on the development of this enzyme. It was found to have low activity in human newborn liver and to rise to adult values between 2 and 15 years (3 values only). The activity of trimethyllysine β-hydroxylase did not change with age (25). Unfortunately, no data are available on the development of the kidney enzyme. In the rat carnitine synthesis from γ-butyrobetain occurs only in the liver. γ-butyrobetain hydroxylase activity increases after birth with adults values being attained on about the 8th postnatal day. No activity was found in other tissues (placenta, kidney, brown adipose tissue (26). During these 8 days the carnitine content of the rat's body increase from 15.6 ± 0.54/g to 27 ± 1.32 nmoles/g (26).

Since carnitine is essential for optimum fatty acid oxidation and since the rate of this process is low in fetal rat and probably also in other mammalian fetuses, it was asked how far carnitine can cross from mother to fetus. In the rat transport of carnitine is slow (7), in sheep it is even slower, while in guinea-pigs carnitine is transferred very rapidly from mother to fetus (27). No direct data are available for man, but usually levels of both free and acetylcarnitine are slightly higher in the fetal than the maternal blood (28) or the same (29) suggesting that transfer is adequate. If we accept that the blood levels of acetylcarnitine and of γ-hydroxybutyrate reflect hepatic oxidation of fatty acids, then in all species, except rabbits, delivery triggers fatty acid oxidation and ketone formation. Data for rabbits are equivocal and more data are required before a definite conclusion is made.

An interesting and unsolved problem is the role of carnitine actyltransferase (CAT), which as the name implies transfers acetylcarnitine (and perhaps acetoacetyl), β-hydroxybutyryl and other short chain acid carnitines across the mitochondrial but also the peroxisomal and perhaps microsomal membranes (30). Mitochondrial activity in both liver and brown fat increases considerably after birth (31). However, activity in the hepatic microsomes, which presumably includes peroxisomes is much lower in the suckling period and rises only when weaning has commenced, suggesting that the role of the microsomal enzyme is not related to fatty acid oxidation. In contrast to the liver, CAT activity in brown fat microsomes increases postnatally, even though it is always lower by a factor of 3 than in the mitochondria. Very recently it has been suggested that peroxisomal

and DL-carnitine are well tolerated by both man (44) and mammals (15).

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REVIEWS OF BOOKS

Nutritional and Diet Therapy in Gastrointestinal Disease

Martin H. Flock. Plenum, New York, pp 480, 1981. Price $35.00.

This is a single-authored book based on 25 years of cummulative experience in clinical gastroenterology. Part I deals with the physiology of eating, dietary elements, normal nutritional requirements and assessment of weight loss. Part II deals with nutritional recommendations based on pathophysiology of gastrointestinal, hepatic, biliary and pancreatic disorders. Part III includes chapters on enteral and parenteral nutrition. Thirty-two types of diets with sample menus and food lists are appended.

It is a comprehensive monograph providing practical advice on dietary management of disorders of the gastrointestinal tract. Discussion of the physiological background is very weak and many recommendations are dogmatically presented. Not everyone will agree with the advice proffered but the monograph can be the starting point for gaining initial information about dietary therapy in digestive disorders.

Diet and Resistance to Disease

Marshall Phillips and Albert Baetz, Editors. Plenum, New York, pp 290, 1981. Price $32.50.

The critical role of nutrition in modulating immune responses has been the focus of much recent work. Pioneering studies from India and South Africa showed impairment of cell-mediated immunity in young children with protein-energymalnutrition. This led to many investigations to evaluate the role of individual nutrients in leukocyte function. This publication records the proceedings of a conference organized by the American Chemical Society in which some of the active investigators in the field of nutrition participated. The nutrients discussed include vitamins B, C and E, and trace elements zinc, copper, iron and selenium.

The quality of the contributions varies from fair to poor. Some provide an overview of the topic, others discuss individual experiments and provide actual data. The editing and organization leave much to be desired. There is little logic to the sequencing of various chapters. The book is a poor attempt at summarizing current knowledge of the topic and pales besides other available texts on nutrition and immunity.

Handbook of Nutritional Requirements in a Functional Context

Miloslav Recheigl, Editor, Volumes I and II. CRC Press, Boca, Florida, pp 542, 1981. Price $170.00.

The publisher and editor have undertaken the monumental and perhaps over-ambitious project of bringing together in one publication all the important information in the field of nutrition. Only some sections of the multivolume compendium have been published to date. Topics included in the two volumes under review include the relationship of nutrition to cellular and biochemical differentiation, physiological states, hematopoietic system

endocrines, toxic ingestants, work performance, behaviour and stress. There are 39 chapters contributed by 49 authors. The extent of coverage, length, and style of different contributions vary enormously. For example, the chapter on nutrition and differentiation is mainly made up of 39 tables with 750 references, whereas the section on acclimatization to heat is a concise narrative of 5 pages and 9 references without any tables or figures. Erythropoiesis is described in 13 pages with 121 references; leukopoiesis gets 100 pages with 721 references. One doubts if these stark differences in style and length reflect any editorial direction. Many of the manuscripts were submitted almost 5-7 years before publication and thus recent data and references are lacking. It is ironic that the massive multivolume series has been entitled a "handbook". Institutions and most certainly individuals, will get better value for their money if they look elsewhere for the same fund of information.

Elemental Diets

Robin I. Russell, Editor. CRC Press, Boca Raton, Florida, pp 261, 1981. Price $74.95.

Malnutrition may arise from a primary deficiency of nutrient intake or occurs secondarily to many diseases of the gastrointestinal tract and other systems. Nutritional support of malnourished patients reduces the incidence and severity of complications, particularly sepsis. One of the modalities of providing nutritional supplements involves the use of elemental diets. This book includes sections on nutrient absorption, assessment of nutritional status, formulations of elemental diets and their clinical use in various medical and surgical conditions. The summary chapter discusses the value of elemental diets in relation to other nutritional aids. Most of the chapters are well written. The 5-page contribution on causes and results of nutritional insufficiency is very poor and superficial. Bibliography dates to 1979 and earlier, a reflection perhaps of prolonged gestation period. Despite these limitations, the volume is a welcome, although overpriced, addition to the growing literature on nutritional support.

Evaluating the Impact of Nutrition and Health Programs

Robert E. Klein, Merrill S. Read, Henry W. Riecken, James A. Brown, Jr., Albert Pradilla and Carlos H. Daza, Editors. Plenum, New York, pp 462, 1979. Price $29.50.

Recognition of the enormous public health importance of nutritional problems in the Third World led to many attempts at nutritional supplementation and health service support. Surplus food from different countries has been shipped to those areas of the world where malnutrition is rampant. However, there is scanty information on the immediate and long-term evaluation of such attempts. The Pan American Health Organization brought together a group of experts to prepare a comprehensive review of the subject, particularly relationships between a variety of services and interventions, the causal role of social, economic and enviromental factors, and practical methods for evaluation of program impact. The attempt succeeds only partially.

The first section deals with conceptual issues and field data collection problems. The second section includes discussion of the theoretical relationship between program and objective, the role of family composition and structure in modulating impact and the influence of health and nutrition on household economics and social competence. The third section deals with practical organizational issues and dissemination of information.

While it is a good state-of-the-art review and emphasizes the

difficulties involved, the book fails to provide a practical guide to the evaluation of nutrition and health interventions.

Nutrition and Food Science

Walter Santos, Nabuco Lopes, J.J. Barbosa, Dagoberto Chaves and Jose Carlos Valente, Editors. Volumes 1, 2 and 3. Plenum, New York, pp 808,850 and 815, 1980. Price $195.00.

The 3-volume set records most of the symposia held during the Rio de Janero International Congress of Nutrition. The contents reflect the quality of academic data presented but cannot paint the tremendous socio-cultural success of the Congress.

Breast Feeding and Supplementary Foods

Aree Valyasevi and Jean Baker, Editors. pp 76, 1980. Gratis.

This slim publication records extended summaries of papers given during a workshop held in Bangkok, November 17 - 18, 1979. Topics covered include present knowledge of human milk composition and health benefits, immunological aspects, promotion of breast feeding, and country reports from Bangladesh, Burma, India, Indonesia, Malaysia, Nepal, Pakistan, Papua New Guinea, Philippines and Sri Lanka. Complimentary copies are obtainable by writing to Professor Aree Valyasevi, Ramathibodi Hospital, Rama VI Road, Bangkok, Thailand.

NUTRITION RESEARCH

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EDITORIAL

Poverty Amidst Plenty: The Enigma of Malnutrition in Affluent North America

The familiar picture of young children with marasmus and kwashiorkor in countries with poor economic resources and inadequate agricultural and industrial development is perhaps understandable. Economic constraints are bound to reduce food availability and nutrient intake. In addition to these factors, maldistribution of food at different levels including intra-familial sharing, increases the chances of malnutrition. It has been estimated that several hundred million individuals suffer from protein-calorie malnutrition and deficiencies of vitamin A, iron and other essential nutrients worldwide. A survey of several Central and South American countries revealed malnutrition to be the primary or major contributing factor in the death of more than one-half of children under five years of age. Similar data are available from several other regions of the world.

The prevalence of nutritional deficiency states in affluent North America is unexpected and shocking. In the United States and Canada, nutrition surveys of randomly selected subjects in the general population have revealed evidence of nutritional deficiencies as judged by nutrient intake data, physical signs and biochemical analyses. The prevalence of iron and folate deficiency, both obvious and subclinical, in certain groups was as high as 25 percent of the study population. Of course, one might question the biological relevance of slight reduction in nutrient intake when the reference yardstick of Recommended Dietary Allowances itself has less than a rocky base. The genesis of malnutrition in affluent North America is ill understood. A group specially at high risk of developing nutritional problems are the elderly. Economic problems, changes in taste acuity, physical isolation and depression, and gastrointestinal changes may contribute to malnutrition in old age. With advancing age, there is a higher occurrence of chronic diseases in some of which dietary factors play an etiologic role. Infection and cancer are common in the elderly and are examples of syndromes that can both be the cause and the result of nutritional problems. The interrelationship between nutrition and immunocompetence in the elderly is examined in a paper elsewhere in this number of *Nutrition Research*.

Protein-calorie malnutrition is being increasingly recognized among hospitalized patients of all age groups. The prevalence of nutritional problems varies with the population being studied but the general impression is that of all the patients referred to nutrition support teams in a general hospital, more than one-half of the subjects are over the age of 60 years. The location of the institution and its population base, its level of care in terms of primary, secondary or tertiary, the nature and severity of the primary disorder that prompted attendance, nutritional and therapeutic management, and other less obvious determinants are important factors. An article in this issue examines these problems in two hospitals in the same city.

St. John's, Newfoundland Ranjit Kumar Chandra

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NUTRITIONAL STATUS OF ADULT PATIENTS IN TWO ACUTE CARE HOSPITALS: A UNIVERSITY HOSPITAL AND AN AFFILIATED VETERANS ADMINISTRATION MEDICAL CENTER

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ABSTRACT

Paired surveys designed to assess the nutritional status of adult inpatients were conducted in two acute care medical centers: University of Maryland Hospital (UMH) and its affiliated Veterans Administrtion Medical Center (VAMC). Nutritional profiles of the patients were determined by anthropometric measurements: measured height and weight, triceps skinfold (TSF), and mid-arm muscle circumference (MAMC), and by a chart review. The criteria used to define severe deficits were less than 80% for ideal body weight (IBW) and MAMC and less than 50% for TSF of sex specific standards. A serum albumin of 2.8g/100ml or less was regarded as a severe deficit. Chart review revealed missing admission weights in UMH and VAMC charts in 40% and 65% and missing heights in 60% and 88%, respectively. The VAMC had a greater percentage of patients that were below standards for IBW ($p<.005$) and TSF ($p<.005$). However patients at UMH without insurance were not significantly different from VAMC patients in any measured parameter. Patients with Third Party Payment coverage had significantly better nutrition profiles in all parameters ($p<.01$) than the VAMC and UMH patients without insurance. Patients from both medical centers were combined to compare the nutritional profiles of medical vs. surgical patients and of various disease classifications. Medical patients were not significiantly different from surgical patients in any anthropometric measurement. However, medical patients had more significant deficits in serum albumin ($p<.005$) than surgical patients. Thirty-six percent of the total medical center population had evidence of protein-calorie malnutrition (PCM) and only 19% of these malnourished patients were receiving physician ordered supplements. Patients diagnosed with cancer, cardiovascular or pulmonary disease were at significant risk for PCM when compared to the rest of the patient populations ($p<.01$).

KEY WORDS: Protein-calorie malnutrition, anthropometric measurements, somatic protein, visceral protein, marasmus, kwashiorkor.

INTRODUCTION

Nutritional assessment surveys have identified a high prevalence of protein-calorie malnutrition (PCM) among hospitalized medical and surgical patients (1-6). Such surveys have mainly consisted of anthropometric measurements and chart reviews for laboratory data such as serum albumin levels and total lymphocyte counts (1,3,7-9). The loss of lean body mass, the depletion of calorie reserves and the impairment of biochemical function due to malnutrition should not be accepted as part of the disease process. Aggressive nutritional support can reverse PCM and may result in better patient response to treatment, shorter hospital stays, and a decreased risk of mortality and morbidity (1). Nutritional assessment data can be used by health care providers to assess the need and timing of nutritional support and to provide guidelines for initiating nutritional therapy and for monitoring changes in the nutritional status of the patients.

This study was undertaken to compare the extent and severity of PCM among inpatients at the two acute care hospitals: a large urban university hospital and its affiliated Veterans Administration Medical Center. In the latter hospital, there was a predominantly male population of lower economic status and with a frequent history of alcoholism. Other objectives of this study were: 1) to see if pertinent nutritional indices were available in the medical records; 2) to examine the relationship of financial status and admitting diagnosis to the incidence of PCM and; 3) to see if a relationship exists between deficits in serum albumin and deficits in anthropometric parameters among our patient groups.

METHODS

Paired surveys designed to assess the nutritional status of adult inpatients were conducted over two weekends at the University of Maryland Hospital (UMH) and the Baltimore (Loch Raven) Veterans Administration Medical Center (VAMC). Nutritional profiles of the patients surveyed were determined by anthropometric measurements and by chart review. Teams of dietitians and physicians measured the patients for height, weight, triceps skinfold thickness (TSF) and mid-arm circumference. Mid-arm muscle circumference (MAMC) was derived using the formula: mid-arm circumference - .314 x TSF (3).

One member of each team performed all measurements, one member recorded the results, and one member recorded the chart review information. Lange skinfold calipers, (Cambridge Scientific Industries, Inc., Cambridge, Maryland), Ross Laboratories measuring tapes (Columbus, Ohio), and Detecto balance scales (Detecto Scales, Inc., Brooklyn, New York), were used to take measurements. To insure standardization, each survey team was previously trained in taking anthropometric measurements and recording survey information at a nutritional assessment workshop.

All patients were measured in pajamas without shoes. At UMH when height could not be measured in the conventional manner it was determined by taking a linear measurement with a measuring tape from crown to heel. Anthropometric skinfold measurements were done according to the technique outlined by Jelliffe (10). Data recorded from the chart included date of admission, medical or surgical admission, admitting diagnosis, financial classification, age, race, sex, admission height and weight and the physicians diet order. Serum albumins were collected from UMH records but the chart review for VAMC patients did not include serum albumin results or race.

Sex specific standards for anthropometric measurements used to determine deficits in TSF and MAMC were those of Jelliffe (10). Standards for ideal body weight (IBW) were from the Ideal Weight for Height 1959 Metropolitian Life Insurance Standards corrected to nude weight without shoes and heels (10). Severe deficits were defined as: <80% standard for MAMC, and IBW, <50% standard for TSF, and <2.8g/100ml for serum albumin. A moderate deficit was defined at 80% - 90% standard for MAMC, and IBW, and 50% - 70% of standard for TSF and 2.8 - 3.5 g/100ml for serum albumin.

Patients were labelled severely protein calorie malnourished if they had a severe deficit in MAMC and a severe deficit in either or both IBW and TSF. Patient profiles which included one moderate and one severe deficit (one of which was for MAMC) or profiles with two moderate deficits (one of which was for MAMC) were noted as having moderate PCM. Patients were classified according to ten disease categories: cardiovascular, cancer, central nervous system, gastrointestinal, infectious, renal, trauma, pulmonary, alcoholic, and miscellaneous (endocrine, hematology, collagen disease). Each diagnostic category was compared to the rest of the hospital population to determine if that patient group had statistically significant differences in the incidence of PCM or in the incidence of specific deficits in anthropometric parameters and serum albumin. Cancer patients were excluded from statistical analysis in the general hospital population, because a high percentage of cancer patients are known to be at severe nutritional risk (4).

All mathematical computations and statistical analysis were processed by a computer which used the health program of the Statistical Package for the Social Sciences (11). Statistical analysis was based on chi square analysis. The level of significance was taken at $p<.05$.

RESULTS

The UMH population (n=395) included 229 males and 166 females of whom 158 were not receiving third party payment. The VAMC population (n=173) was comprised of 171 males and 2 females. The combined study population ranged in age from 19 to 93 years with a mean ± standard deviation age of 53 ± 17.54 years.

Twelve patients at VAMC and 47 patients at UMH were not measured due to either physician or patient refusal or unavailability of the patient at the time of the team's visit. One-hundred-thirty-one patients could not have heights and/or weights measured due to a physician order of strict bed rest. Thirteen of these patients had anasarca noted on clinical examination and were excluded from statistical analysis.

Table 1 shows the number and percentage of patients with moderate and severe deficits in anthropometric measurements for each hospital. Twenty-eight percent of the combined hospital population had moderate or severe deficits in IBW; 54% of the population had deficits in TSF and 36% had deficits in MAMC. There was a significantly greater percentage of deficits for IBW and TSF in VAMC patients ($p<.005$). However, there was no significant difference for MAMC deficits between the two hospitals. At both hospitals, there were no significant differences between medical and surgical patients in any measured anthropometric parameter.

Table 2 shows the incidence and degree of PCM at the two hospitals. Thirty-six percent of the combined population were at risk for PCM. The likelihood of PCM was significantly greater for VAMC patients (41%) than for UMH patients (33%) ($p<.05$). There was a significantly greater percentage of

TABLE 1

Deficits in Anthropometric Measurements for University of Maryland Hospital (UMH) and the Veterans Administration Medical Center (VAMC)

Anthropometric Measurement	Total Number Patients	Number with Moderate Deficit (+)	%	Number with Severe Deficit (++)	%
Ideal Body Weight (IBW)					
UMH	315	52	17	26	8
VAMC	122	21	17	24	20
UMH + VAMC	437	73	17	50	11
Triceps Skinfold (TSF)					
UMH	348	109	31	71	20
VAMC	161	47	29	45	28*
UMH + VAMC	509	156	31	116	23
Mid-Arm Muscle Circumference (MAMC)					
UMH	348	71	20	46	13
VAMC	161	40	25	24	15
UMH + VAMC	509	111	22	70	14

* $p<.005$ UMH versus VAMC patient population

(+) Moderate Deficit: 80-90% of standard for IBW and MAMC, and 50-70% of standard for TSF.

(++) Severe Deficit: <80% of standard for IBW and MAMC, and <50% of standard for TSF.

patients at VAMC (32%) with moderate malnutrition ($p<.05$). Of the 155 moderately and severely malnourished patients, only 29 (19%) were receiving physician ordered tube feedings, oral nutrition supplements or were being parenterally hyperalimented.

The population was further divided into disease categories. Table 3 shows the incidence of moderate and severe malnutrition by diagnosis for the combined hospital population. In the combined population, those patients with cancer, cardiovascular and pulmonary disease had a significantly greater amount of PCM than the rest of the hospitalized patients ($p<.01$). Substandard values for all three anthropometric measurements were present in all disease categories. In comparison to the rest of the hospital population, those patients with the diagnosis of cardiovascular disease, cancer and pulmonary disease had a significantly greater amount of moderate and severe deficits for IBW ($p<.05$). In addition, patients in these disease categories had significantly more deficits in MAMC ($p<.01$). Among the patients admitted with trauma and cardiovascular disease, there were significantly greater severe deficits in TSF ($p<.01$, $p<.05$) and moderate deficits in TSF for patients with gastrointestinal disease ($p<.01$). Alcoholic patients had a significant amount of moderate and severe deficits for TSF ($p<.005$, $p<.001$).

Medical patients at UMH had significantly greater deficits in serum albumin than surgical patients ($p<.005$). However, this study found no significant relationship between serum albumin and deficits in anthropometric measurements.

TABLE 2

Prevalence and Degree of Protein Calorie Malnutrition (PCM) at the University of Maryland Hospital (UMH) and the Veterans Administration Medical Center (VAMC)

Degree of PCM	UMH		VAMC		Both Hospitals	
	Number of Patients	Percentage	Number of Patients	Percentage	Number of Patients	Percentage
Moderate PCM	64	20*	39	32*	103	24
Severe PCM	41	13	11	9	52	12
Total	105	33	50	41	155	36

PCM: Simultaneous deficits in MAMC and either/or both IBW and TSF.

Moderate PCM: Two moderate deficits or one moderate and one severe deficit (one of which was MAMC)

Severe PCM: Severe deficit in MAMC and severe deficit in IBW and/or TSF.

* $p<.05$ UMH versus VAMC patient population

TABLE 3

Prevalence of Moderate and Severe Protein Calorie Malnutrition (PCM) by Diagnosis Among the Patient Population of the University of Maryland Hospital and the Veteran's Administration Medical Center Combined

Diagnosis	Total Number of Patients	Moderate PCM		Severe PCM	
		Number of Patients	Percent	Number of Patients	Percent
Cardiovascular*	82	9	11	7	9
Cancer**	80	6	7	31	39
Central Nervous System	42	10	24	6	14
Gastrointestinal	48	3	6	14	29
Infectious Disease	24	3	12	4	17
Renal	26	4	15	5	20
Trauma	39	8	21	9	23
Pulmonary*	18	2	11	8	45
Alcoholism	37	1	3	6	16
Miscellaneous	41	6	15	13	32
Total	437	52	12%	103	24%

* $p<0.01$, ** $p<0.001$

Miscellaneous category included: Endocrine, Hematologic, Collagen, Dermatologic, Rheumatologic and Psychiatric diseases.

Diagnostic categories were compared to the total population minus cancer patients.

Patients with the primary diagnosis of cardiovascular, pulmonary and renal disease were noted to have a significantly high pervalence of deficits in serum albumin in comparison to the rest of the hospital population ($p<.05$).

UMH patients not receiving third party payment were not significantly different from VAMC patients in any nutritional parameter. However, patients at UMH with third party payment coverage had significantly better nutrition profiles in all parameters than either the patients at the VAMC or the patients at UMH without insurance ($p<.01$).

Patients over the age of 60 were more likely to have deficits in MAMC ($p<.01$). At UMH, female caucasions had significantly greater prevalence of deficits in MAMC ($p<.01$), while black males had a greater prevalence of deficits in TSF ($p<.01$).

Medical charts were reviewed to determine the availability of recorded information. This survey revealed missing admission weights in 49% of UMH's medical records and in 65% of VAMC's charts. Sixty percent of UMH patients' records and 88% of VAMC's charts had missing heights. At UMH, serum albumin was available and recorded in only 59% of the charts.

DISCUSSION

For any patient population a nutritional profile can be determined if sufficient information is available in the medical record and if anthropometric measurements are performed accurately. When the profile is compared to "standards of reference", conclusions regarding type of malnutrition can be drawn and nutritional therapy can be prescribed. Marasmus is frequently diagnosed in the adult hospitalized patient because of clinically evident weight loss, fat and muscle wasting and dietary evidence of inadequate oral intake. However, the kwashiorkor patient may be overlooked because these patients may appear well nourished due to edema, ascites or obesity (3,4). Biochemical protein status evaluation (e.g. serum albumin measurement) is essential in making the diagnosis of kwashiorkor (12), and this information was available in only 60% of UMH charts.

Our study showed a lack of recorded nutritional indices in the medical record. It was very difficult to find heights and weights in the chart and, when recorded, they were not consistently found in any one location. Moreover, it is probably from questioning patients that many admission heights and weights had been taken from the patients history and were not measured data. Without accurate baseline data on admission height and admission weight, a statement regarding patient response to hospitalization therapy as measured by a change in weight over time cannot be determined. Several studies support our findings of missing height and weights in the chart. Prevost and Butterworth found heights missing 56% and weight in 23% of their hospitalized patients surveyed (13). Tobias found as much as 96% missing of either height or weight (14). Hill found that IBW could be determined for only 15% of his studied hospital population (15).

Standards for TSF and MAMC in this survey were that of Jelliffe. Jelliffe's standards are currently the most widely used in assessing nutritional status of hospitalized patients. These standards are not ideal as they are not age specific and were derived from different populations at different times by differnet observers, using different techniques and with variable apparatus (10,16,17). Burgert compared Jelliffe's standards to the reference standards outlined in the National Health Survey and the Ten State Survey and found

Jelliffe's standards to be significantly higher for men's TSF and women's MAMC and significantly lower for women's TSF and men's MAMC ($p<.05$). (7). Literature review does not provide race specific standards or standards for the elderly. This survey identified black patients at UMH as having greater deficits in TSF and patients over the age of sixty having significant deficits in MAMC ($p<.01$). For future surveys, it may be more appropriate that the age and sex specific standards compiled by Frisancho in 1974 based on the United States Ten State Nutrition Survey be used as the point of reference for TSF and MAMC (7, 12,18). Racial standards remain to be derived.

It is difficult to compare the incidence of PCM from one nutritional assessment survey to another survey. There is no set classification to rank severity of malnutrition. Arbitrary levels of nutritional deficits have been set by different investigators and institutions. Bistrian and Blackburn defined moderate malnutrition as 60 to 90% of standard in any one nutritional parameter and severe malnutrition as less than 60% of standard for any one nutritional parameter (4,5). Using their criteria for malnutrition 49% of our combined UMH, VAMC patients would be considered moderately or severely malnourished. Our study found 36% of the combined UMH, VAMC population to be at moderate or severe risk for PCM. This lower percentage is the reflection of a more stringent definition of PCM. Patients at nutritional risk in our study had a composite of two or more moderate or severe deficits in anthropometric measurements.

Blackburn and Bistrian found medical patients to have lower fat stores and better protein reserves than surgical patients (4,5). However, Bollett found no difference in nutritional status between patients on the medical service and those patients on the surgical service (2). Similarly, our survey showed no significant difference in anthropometric measurements between medical and surgical patients. In contrast to Bistrian and Blackburn, our study at UMH found medical patients to have significantly lower serum albumins than surgical patients.

When the combined medical center populations were categorized according to diagnosis, abnormal values for all three anthropometric values and serum albumin were present in all categories. This result is consistent with the findings of Blackburn and Bistrian (4,5). In our study, patients diagnosed with cardiovascular disease, (omitting those with anasarca), pulmonary disease or cancer were found to be at significant risk for PCM when compared to the rest of the medical center population ($p<.01$). These three disease categories had simultaneous severe deficits in IBW and MAMC and deficits in serum albumin.

Our survey is in agreement with the following findings determined by Bollet and Owens: i) there was no significant difference in substandard IBW between sexes in the hospital populations; ii) pulmonary and congestive heart failure patients had an increased frequency of hypoalbuminemia; iii) one-hundred percent of renal patients had serum albumins that were substandard; iv) gastrointestinal and alcoholic patients had a greater degree of significant deficits in TSF when compared to the rest of the hospitalized population ($p<.01$) In addition, our study found trauma and cardiovascular patients to have significant deficits in TSF ($p<.01$, $p<.05$). Our survey did not support Bollet's results that alcoholic patients had the most severe deficits in serum albumin although deficits in albumin for the alcoholic patients studies approached significance ($p<.06$) (2). Moreover, our study did not support Bistrian's results that showed a correlation between MAMC and serum albumin for general hospitalized patients (4,5).

This survey supports the logical reasoning that patients who do not have third party payments are more likely to have inadequate protein and calorie

intake and concomitant nutritional problems. The VAMC had significantly more patients with PCM than UMH ($p<.05$). This higher incidence of PCM might be explained by the greater number of patients at VAMC who are of low economic status and were admitted to the hospital with the diagnosis of alcoholism.

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NUTRITION AND IMMUNOCOMPETENCE OF THE ELDERLY: EFFECT OF SHORT-TERM NUTRITIONAL SUPPLEMENTATION ON CELL-MEDIATED IMMUNITY AND LYMPHOCYTE SUBSETS*

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ABSTRACT

Fifty-one subjects above the age of 60 years were examined. Assessment of immunocompetence showed a progressive age-related decline in cell-mediated immunity and marked reduction in serum thymic factor activity. Twenty-one (41%) individuals showed clinical, anthropometric, biochemical or hematologic evidence of nutritional deficiency. In these patients, there was evidence of reduced number of rosette-forming T lymphocytes, both T4+ and T8+ subsets, especially the latter. The role of nutritional factors in contributing to impaired immunity was evaluated by providing oral supplementation of approximately 500 Kcal per day for 8 weeks in a formula containing proteins, fats and carbohydrates as well as minerals, vitamins and trace elements, and reassessment of immunocompetence. Nutritional therapy resulted in a marked improvement in clinical and biochemical indices of nutrition, T lymphocyte subpopulations and cell-mediated immunity. The results indicate that malnutrition is a frequent problem in the elderly and contributes to reversible impairment of immunocompetence.

KEY WORDS: Old age, nutritional deficiency, immunocompetence, protein-calorie malnutrition, T lymphocyte subsets, monoclonal antibodies, cell-mediated immunity, thymic factor, zinc, iron, ageing.

INTRODUCTION

All men die and those who escape genetic mutations and the biologic and physical hazards of childhood and maturity begin, usually in their seventh decade, to lose physical power, mental agility and resistance to disease. Each passing year brings a higher statistical chance of death. There is no satisfactory explanation of the entire process of ageing. In some species, there is no evidence of ageing, there being an exponential death rate by predation or

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accident. In man, ageing is associated with atrophy and loss of cells and restricted ability to repair what is damaged or lost. The pattern of diseases of the elderly (infection, cancer, autoimmunity, amyloidosis) resembles that seen in primary immunodeficiency states, points to progressive decline of immunocompetence. At the same time, there are alterations in nutritional status and body composition, including decreased lean body mass, loss of visceral protein and increase in the relative proportion of body fat (1). These changes in body constituents may result from a variety of pathogenetic factors, such as altered taste acuity, reduced food intake, malabsorption and the metabolic consequences of concurrent disease.

The inevitability of senescence and death has intrigued scientists and philosophers; more recently, it has provided a challenge to nutritionists and immunologists. Among a group of 51 subjects above the age of 60 years, we found evidence of nutritional deficiencies in 21. We studied the nutritional and immunologic status of these 21 malnourished elderly individuals before and after 8 weeks of dietary supplementation using a commercial liquid formula.

SUBJECTS AND METHODS

In a survey of 51 individuals above the age of 60 years, who were free living by themselves or with their families, 21 were found to have evidence of protein-calorie malnutrition on the basis of clinical features, anthropometric and biochemical assessment. A careful medical history was recorded as well as data on 24-hour dietary intake. Systematic physical examination was conducted to exclude serious systemic disease, such as cancer, chronic inflammatory bowel disease and liver and kidney disorders. Anthropometric measurements included weight, height and skin-fold thickness. The studies were repeated after 8 weeks of supplementation with a commercial liquid formula (ENSURE, Ross). The average daily consumption was 471 ± 30 ml which provided approximately 500 Kcal. Each 100 ml of the formula contains protein 3.72g, fat 3.72g, linoleic acid 2.00g, carbohydrate 14.50g, vitamin A 264.00 IU, vitamin D 21.00 IU, vitamin E 1.60 IU, vitamin K_1 0.10mg, vitamin C 6.40mg, folic acid 0.02mg, vitamin B_1 0.16mg, vitamin B_2 0.18mg, vitamin B_6 0.16mg, vitamin B_{12} 0.63mcg, niacin 2.10mg, choline 0.05g, biotin 0.02mg, pantothenic acid 0.53mg, sodium 0.07g, potassium 0.12g, chloride 0.11g, calcium 0.05g, phosphorus 0.05g magnesium 0.02g, iodine 0.01mg, manganese 0.21mg, copper 0.11mg, zinc 1.60mg and iron 0.95mg.

Hematology

Hemoglobin and red cell indices were obtained on a Coulter counter. The total white blood cell count was similarly estimated and differential leukocyte count was calculated by examining 200 cells on a Giemsa-stained blood film. Red cell morphology was noted. Serum ferritin was estimated by double antibody radioimmunoassay (GammaDab, Clinical Assays). Serum concentrations of vitamin B_{12} and folate were estimated by using a dual radioassay kit (Amersham).

Biochemistry

Serum concentrations of albumin, prealbumin, transferrin and retinol-binding proteins were estimated by turbidometric method using a laser nephelometer and monospecific antisera (Hyland). Zinc was estimated by atomic absorption spectrophotometry.

Immunology

Peripheral blood mononuclear cells were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation. Interface cells were collected, washed three times with Hanks' balanced salt solution, and then resuspended at a concentration of $2x10^6$ cells per ml in RPMI 1640 medium, containing glutamine (2mM), penicillin (100 U per ml) and streptomycin (50 ug per ml), and 10 percent fetal-calf serum. Adherent cells were removed by overnight incubation in 60x15 mm petri dishes at 37°C in an atmosphere of 95 percent air and 5 percent carbon dioxide. Rosette-forming T cells: Lymphoid cells were mixed with equal volume of 1% neuraminidase-treated sheep red blood cells (SRBC) in 10 percent fetal calf serum. The mixture was incubated at 37°C for 5 minutes, centrifuged at 200 g for 5 minutes and incubated again at 4°C for 10 minutes and examined for the proportion of cells forming rosettes with SRBC. T lymphocyte subsets: Three monoclonal antibodies with specificities for T3, T4 and T8 cell subpopulations were used. The antisera were reactive by complement-mediated cytolysis and/or indirect immunofluorescence to a titer from 1: 100,000 to 1: 500,000. Cytofluorographic analysis of lymphocytes was performed by indirect immunoflourescence with fluorescein-conjugated goat anti-mouse IgG on System 50H Cytofluorograph Cell Sorter. B lymphocytes: Cells bearing surface membrane immunoglobulin (smIg) were recognized by speckled or crescent-shaped fluorescence on staining with fluorescein-conjugated goat IgG $f(ab')_2$ directed against human IgG, IgA and IgM heavy chains. Mononuclear cells were incubated in culture medium at 37°C for one hour and then incubated with the conjugated antisera. After thorough washing, the cell preparations were examined under a fluorescence microscope equipped with epi-illumination. Lymphocyte proliferation: Lymphocyte stimulation response to phytohemagglutinin (PHA) was evaluated as a dose response curve using PHA in concentrations of 5,10 and 15µl volume, as previously described (2). Thymic factor activity: Thymic factor (TF) activity was quantified by the modified method of Dardenne and Bach (3) as described earlier (2,4). The blood was allowed to clot at 4°C in plastic centrifuge tubes. The serum was separated in a refrigerated centrifuge and filtered immediately. TF activity was assayed either on the same day that the blood was collected or on samples kept frozen at -40°C for up to one week. Spleen cells were obtained from 8 to 12 week-old male C57Bl/6J mice that had been thymectomized 10 to 20 days before they were killed. The spleen cells were dissociated, washed twice in Hanks' solution, resuspended in a concentration of $30x10^5$ cells/ml, and incubated at 37°C with serial $\log_2$ dilutions of test sera in Hanks' medium and azathioprine (10 ug/ml). After 80 minutes, a 1 percent suspension of sheep red blood cells (SRBC) was added. SRBC had been kept in Alsever's solution for up to three weeks and before use were washed twice with saline and twice with Hanks' solution. The cell suspension was centrifuged at 4°C for five minutes and resuspended gently. Rosettes were observed in a hemocytometer. The highest dilution of test serum sample that was able to restore azathioprine sensitivity, i.e. induce 50 percent inhibition of SRBC rosette forming cells, was taken as the concentration of TF activity in the serum.

Statistics

Differences between groups were analyzed by the Student's two-tailed t test. Comparisons between results on individual subjects before and after nutritional supplements utilized paired t test.

RESULTS

Results of cell-mediated immune responses, lymphocyte numbers and subsets in elderly individuals are shown in Table 1 and Figures 1 and 2. Impaired delayed hypersensitivity response or anergy was seen in 15 subjects. There was a progressive decrease in the number of circulating rosette-forming T cells with advancing age, wereas B cell proportion was unchanged (Fig. 1). Thymic factor activity was decreased, particularly in those above the age of 70 years (Fig. 2).

Table 1. Cell-mediated immune responses in the elderly

Age group (years)	Number	Delayed cutaneous hypersensitivity (Number positive)	Lymphocyte stimulation index	Lymphocyte count (per mm^3)
Subjects				
61-70	7	3	43 ± 9*	3,761 ± 382
71-80	8	2	30 ± 7	4,081 ± 317
<80	6	1	21 ± 6	3,465 ± 410
Controls				
21-40**	20	20	159 ± 17	3,946 ± 355

* Mean ± SD

** Healthy adults working in the hospital and laboratory volunteered to provide control samples

The demographic data of the 21 undernourished subjects described in the present paper are given in Table 2. There were 12 men and 9 women. Most of the subjects showed clinical evidence of wasting and 6 exhibited pitting edema on dependent areas. Weight-for-height and skin-fold thickness were reduced. Anemia, defined as hemoglobin concentration less than 11.8 g% and/or reticulocyte response after supplementation was found in 4. Serum ferritin was low in 4 subjects, whereas vitamin B_{12} and folate levels were normal.

The effects of nutritional supplementation on biochemical and immunologic indices are shown in Tables 3 and 4 and in Figures 3 and 4. There was a significant increase in serum levels of various proteins, particularly those with short half-life, namely prealbumin and retinol-binding protein, thus reflecting increased short-term nutrient intake. The mean ferritin concentration rose slightly and in 2 out of 4 iron-deficient individuals, the ferritin level increased to >28 ng/ml. The mean serum zinc levels were significantly higher after therapy (Table 3) but remained <70 µg/100 ml in 4 out of 7 subjects who were considered zinc deficient on first examination.

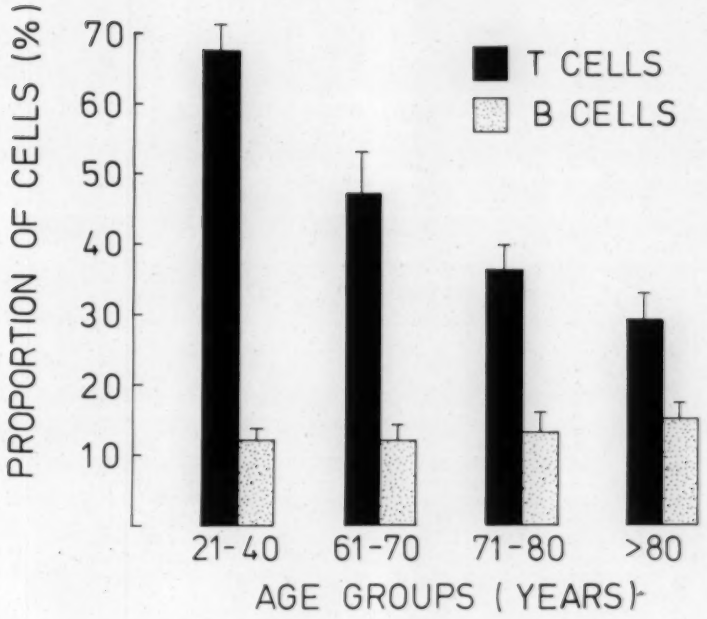

Fig. 1
Age-related changes in the proportion of T and B lymphocytes.

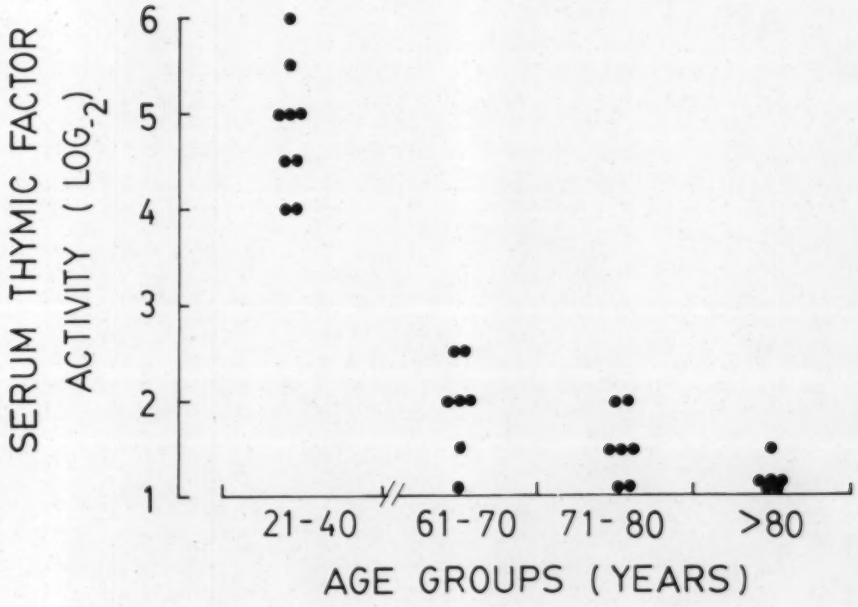

Fig. 2
Age-related changes in serum thymic factor activity.

Table 2. Demographic and anthropometric data and hemoglobin levels.

S. No.	Age	Sex	Weight (lbs)	Height (ft-in)	Skin-fold thickness (mm)	Hemoglobin (g/100 ml)
1	62	M	121	5-8	8.1	12.0
2	64	M	140	5-10	10.0	11.6
3	65	M	126	5-7	9.0	12.8
4	68	M	110	5-6	7-1	13.4
5*	71	M	129	5-7	10.9	10.1
6	73	M	136	5-11	7.1	11.9
7*	76	M	121	5-2	5.8	12.1
8	76	M	132	5-9	4.9	12.8
9	79	M	146	6-0	6.4	13.1
10	82	M	138	5-10	7.1	13.6
11	83	M	129	5-6	8.9	14.1
12*	89	M	118	5-7	5.3	12.0
13	61	F	99	5-2	7.6	11.8
14	65	F	94	4-11	8.1	12.1
15	69	F	112	5-8	6.8	13.6
16*	72	F	124	5-7	7.6	11.2
17*	75	F	139	5-5	8.9	10.8
18	77	F	111	5-7	5.8	12.9
19*	81	F	128	5-5	9.1	13.6
20	87	F	109	5-3	10.8	12.4
21	88	F	101	5-2	6.1	12.1

* Showed evidence of pitting edema.

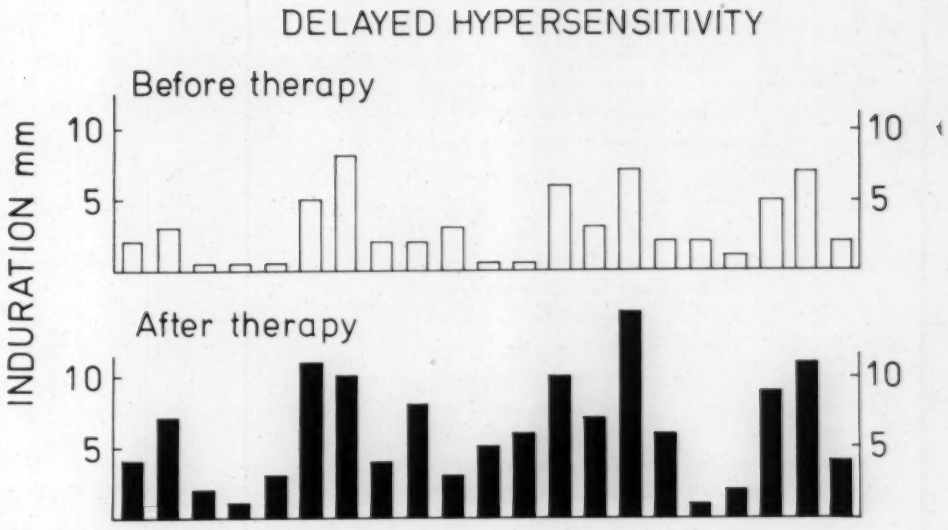

Fig. 3
Maximum diameter of induration in response to intradermal injection of streptokinase-streptodornase in 21 malnourished elderly patients before and after nutritional supplementation.

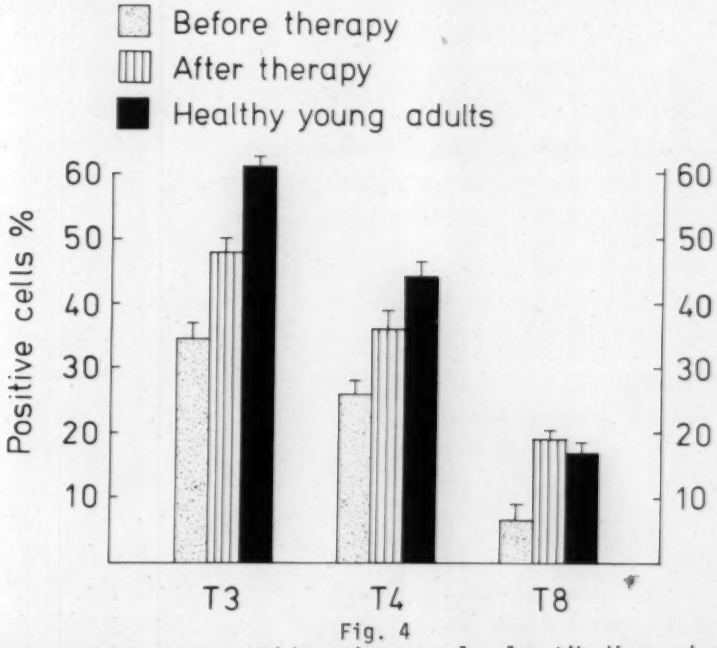

Fig. 4
T lymphocyte subsets enumerated by using monoclonal antibodies and cell sorting technique in malnourished elderly subjects before and after nutritional supplementation

Table 3. Biochemical and hematologic indices.

	Number	Albumin (g/100ml)	Transferrin (mg/100ml)	Prealbumin (mg/100ml)	Retinol-binding protein (mg/100ml)	Ferritin (ng/ml)	Zinc (μg/100ml)
Before therapy	21	2.8 ± 0.6	141 ± 23	12 ± 4	2.3 ± 0.9	92 ± 28*	87 ± 21**
After therapy	21	3.4 ± 0.3	196 ± 37	29 ± 7	5.1 ± 1.1	106 ± 23*	119 ± 17**
p		<0.05	<0.01	<0.001	<0.01	NS	<0.05

Values are given as Mean ± SD

NS Not significant

* Before therapy, ferritin levels were low (<18ng/ml) in three women and markedly reduced (<10ng) in one man. After therapy, two female subjects continued to show iron deficiency.

** Zinc levels were low (<70μg/100ml) in 7 subjects before therapy and in 4 after therapy.

Table 4. Effect of nutritional supplementation on immunologic responses.

	Number	Delayed cutaneous hypersensitivity (Number positive)	Stimulation index*	Rosetting T cells (%)
Before therapy	21	6	38 ± 14	41 ± 7
After therapy	21	12	79 ± 17	57 ± 5
p		<0.05	<0.05	<0.05

Values are given as Mean ± SD

* Maximum response was obtained with 10µl PHA. Results obtained with this amount of PHA are shown.

Immunocompetence improved in many subjects (Table 4). Twelve individuals showed positive delayed cutaneous hypersensitivity reactions. In those who had a positive skin reaction before therapy, the diameter of induration increased on second examination (Fig. 3). There was an increase in the number of rosette-forming T lymphocytes (Table 4) and in the proportion of T3+, T4+ and T8+ cells (Fig. 4). The ratio of T4:T8 cells decreased from 3.7 to 1.9; in healthy young adults, the ratio was 2.6.

DISCUSSION

During the last several years, studies of the effect of ageing on immune response have demonstrated both in vivo and in vitro alterations in the immunocompetence of individuals above the age of 60 years (5-8). Table 1 and Fig. 1 show the age-associated progressive decline in cell-mediated immunity and T cell number. Furthermore, there was a profound reduction of thymic factor activity (Fig. 2). These changes resemble to some extent those seen in protein-calorie malnutrition (9,10). Since the elderly are known to be among the most poorly nourished in industrialized countries and since changes in food intake, body composition and protein metabolism are known to occur with advancing age, the possibility arises that altered immune status in the elderly could be ascribed to nutritional deficiency. The crucial test for this is to evaluate nutritional and immunologic status before and after deliberate supplementation. Our preliminary observations recorded here show that protein-calorie malnutrition was present in 21 (41%) of 51 elderly individuals surveyed and suggest that in these subjects, supplementing the diet with a formula supplying about 500 Kcal extra energy intake per day and containing vitamins, minerals and trace elements resulted in improved nutritional status and immunocompetence.

The study subjects showed clinical, anthropometric and biochemical evidence of protein-energy malnutrition (Tables 2 and 3). There was a reduction in serum levels of albumin, prealbumin, transferrin, and retinol binding protein. In

addition, 7 subjects had low serum zinc levels and 4 showed evidence of iron-deficiency anemia. Cell-mediated immunity was impaired, as judged by reduced number of T cells, particularly T4+ and T8+ lymphocytes, decreased lymphocyte proliferation response to PHA and impaired delayed hypersensitivity skin reactions. After nutritional supplementation, there was a significant improvement both in nutritional and immunologic indices (Tables 3 and 4, Figures 3 and 4).

There are only limited data on immune response of the elderly (5-7). Most studies do indicate impaired immunocompetence. Few have looked at nutrition and immunity at the same time (11). To date, the contribution of altered nutritional status in the elderly to deficits in their immune responses has not been adequately evaluated. Our data indicate that a causal relationship does exist between undernutrition and impaired immunity in many elderly individuals and that this is a correctable abnormality in the majority. It is not clear whether maintenance of good nutrition and improved immunocompetence will alter morbidity and longevity. Obviously, long-term prospective studies are required to answer these questions. These are in progress.

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INTAKE AND ABSORPTION OF NUTRIENTS IN CHILDREN WITH CHOLERA AND ROTAVIRUS INFECTION DURING ACUTE DIARRHEA AND AFTER RECOVERY

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ABSTRACT

The effect of diarrhea on nutrient intake was studied in 19 children with cholera and 13 with rotavirus. The co-efficients of absorption of fat, nitrogen and calories were calculated during the acute and the recovery stages of the disease. Compared to the recovery period, in the acute stage, the intake of nitrogen, fat and calories were reduced by 44%, 32% and 36% in cholera and 22%, 10% and 27% in rotavirus patients respectively. The co-efficient of absorption of nitrogen during the acute stages of both cholera and rotavirus was equally affected, being only 44 and 45% of the ingested amount respectively. In the acute stage, the rotavirus patients absorbed significantly less fat and calories than the cholera patients (44 and 55% in rotavirus versus 72 and 82% in cholera respectively). During the recovery period, the rotavirus patients absorbed a lesser amount of all the nutrients than the cholera patients, suggesting the presence of a longer period of malabsorption in rotavirus than in cholera.

KEY WORDS: Intake of nutrients, co-efficient of absorption, recovery stage of diarrhea, percent reduction of nutrients, acute stage of diarrhea

INTRODUCTION

In developing countries, diarrhea and malnutrition in children are commonly associated (1,2). Martorell et al. (3) have recently reported that "ordinary illnesses" such as common cold or a bout of diarrhea, decreases a child's daily intake of food by 20% and estimated that a malnourished child on an average day consumes 225 calories less than the amount required for optimum growth. Mata et al. (4) in a study in Guatemalan villages have also found that reduction in calorie intake was more pronounced than decrease in protein intake during or after episodes of infectious diseases. In general, little is known about the effect of specific infection on food wastage.

Malabsorption was related to abnormalities in intestinal morphology and was found to be associated with acute bacterial and viral diarrheas (5). Loss of gut enzymatic activities (Na-K ATPase, Disaccharidases) have also been studied in acute diarrhea (6,7). Since the report of Chung et al. (8,9) we have not found any published information on absorption of nitrogen, fat and calories during and after diarrheal illnesses. Rotavirus seems to be the major

cause of diarrheal illness among the under-five population in both the developing and developed countries (10,11). Cholera is still a significant cause of morbidity and mortality in some developing countries (12). The aim of this study was to determine the nutrient intake and estimate the co-efficient of absorption of fat, calories and nitrogen during and after diarrhea due to cholera and rotavirus.

PATIENTS AND METHODS

Thirty-two male children below 5 years of age with histories of acute watery diarrhea and moderate to severe dehydration were selected for the study. After admission into the metabolic study unit of ICDDR, B Dacca Hospital, all children underwent a thorough physical and biochemical examination by the admitting physician and one of the investigators. Patients with pneumonia, frank dysentery and severe malnutrition were not included. Patients, whose condition dictated immediate use of antibiotics were also excluded from the study. Prior approval of the human rights ethical committee and scientific research review committee was obtained for this study. Informed consent was obtained from the parents or the guardian of the patients. Out of the 32 patients studied, 19 had bacteriologically proven cholera and 13 had rotavirus infection, diagnosed by the enzyme-linked immunosorbent assay, ELISA (13).

Treatment of dehydration and subsequent maintenance of hydration were accomplished by the intravenous route using 'Dacca' solution (Composition: Na^+ 133 mmol/L: K^+ = 13 mmol/L; Cl^- = 99 mmol/L and HCO_3^- (as acetate) = 48 mmol/L). After correction of initial dehydration and acidosis, accomplished usually within 4-8 hours, a charcoal tablet was fed and a familiar diet of known composition was offered. The charcoal marker was used to determine the whole gut transit time. The first appearance of the charcoal in the stool was taken as zero time and a second marker was fed at 72 hours after the appearance of the first marker. A metabolic balance was used to take the accurate weight (up to 1 g) of the patient.

Study Diet

The diet was selected after a pretrial study in 20 diarrheal children of the same age group. During this period it was noted that any homogenized diet causes monotony and resulted in a decreased intake even in healthy children. Hence a modified diet was prepared to keep the meal very much familiar and natural to the habitual diets of the children.

The composition of the study diet is shown in Table 1. Two types of diet were used in the present study. A cooked semisolid dish called *khichuri*, made of chicken, lentils and cooking oil was given for lunch and supper. A dessert of *halwa* made of rice, egg, milk, butter and sugar cooked together was offered as breakfast. Bread, banana and cow's milk were offered at specified intervals of time. Though the diets were offered ad libitum, accurate weight was determined subtracting the leftover amount from the amount offered. Breast milk was continued and the actual amount consumed was determined by weighing the babies before and after each breast feeding. A Toledo scale capable of weighing up to 0.1 g was used to measure the weight of food. Samples from each diet were saved and analysed for fat, nitrogen and calories.

D-Xylose Test

In order to compare the absorption of nutrients the d-xylose test was performed on the second day of the study. After fasting from midnight, 5 g of the pentose sugar was fed to the patients in the early morning and one millilitre of blood was drawn after one hour to estimate the blood level of xylose.

Collection of Stool and Urine

Stool and urine were collected separately by using Uribags. Stool collected between two markers were pooled and homogenized in a blender. An aliquot was saved for analysis of fat, nitrogen and calories. Accurate daily intake and output records were maintained during the study period and in case of any vomiting by the patients, the amount was noted accurately. Samples from the vomitus were analysed for nutrient content and subtracted from the intake. Patient's diarrhea usually stopped around 3 to 5 days after admission. The hospitalisation period varied between 7 and 10 days. Two weeks after discharge they were brought back to the hospital and balance studies were repeated.

Serum xylose estimation was done by the Roe and Rice method (14) and fat estimation by the van de Kamer Procedure (15). Total nitrogen content was measured according to microkjeldahl procedure (16). For calorie estimation an adiabetic bomb calorimeter was used, benzoic acid acting as the standard. Percentage reduction in intakes of nutrients (in the acute stage compared to the recovery stage) was calculated as follows: [Intake (recovery)-Intake (acute) x 100]/ Intake (recovery). Co-efficients of absorption of nutrients in both the acute stage and at recovery period were calculated using the following expression: [(Intake - output) x 100]/ Intake.

RESULTS

The clinical and laboratory features of patients with cholera and rotavirus infection are compared in Table 2. Mean age of the cholera patients was higher than that of the rotavirus group. However, the body weights of the two groups of patients were not significantly different. This might be due to the fact that the cholera patients were more malnourished and their growth more stunted than the rotavirus group.

The study here compares cholera and rotavirus patients with regard to their intake and absorption of nutrients during acute diarrhea and after recovery. The comparison group differs in its age and nutritional status and thus some might argue about the validity of strict comparison between these two groups. However, all the results of intake and absorption were expressed in standard units and their tentative comparison is probably not unjustified. Despite these limitations, the study attempted to answer the questions posed, that is 1) to see how intake of food is affected by acute infectious diarrhea and, 2) to estimate the absorption of nutrients during diarrhea.

Mean intakes of nitrogen, fat and calories were calculated in both the acute and recovery period of illness (Table 3). Intakes of all nutrients were significantly reduced (paired t test) during acute cholera. The difference was not significant in rotavirus diarrhoea. Compared to the cholera patients, intakes of all nutrients in rotavirus patients during recovery were significantly reduced (nitrogen $P<0.01$, fat $P<0.05$ and calories $P<0.05$, by Student's t test). The co-efficients of absorption of nitrogen, fat and calories are shown in Table 4. During the acute stage, both cholera and rotavirus patients absorbed less than half of the total ingested nitrogen (44 and 45%); rotavirus patients absorbed considerably less fat and calories than the cholera patients (42 and 55% versus 72 and 81% respectively). During the recovery stage absorption of nitrogen, fat and calories were respectively 78, 89 and 91% in cholera versus 69, 79 and 84% in rotavirus.

Serum xylose levels were measured during the acute stage and after recovery (Figure 1). Patients absorbing less than 20 mg% of xylose were defined as xylose malabsorbers. On an average 72% of patients were xylose malabsorbers in the acute stages of diarrhea but this reduced to 13% in the recovery stage.

TABLE 1

Nutrient Contents of the Study

Diet Offered Daily

Dietary routine	Diets	Nutrient Contents N_2(g%)	Fat (g%)	Cal (K Cal/g)
1. Breakfast (7 a.m.)	Bread	1.29	2.55	3.01
	Halua	0.43	3.58	1.47
2. Lunch (12 noon)	*Khichuri*	0.41	1.75	0.94
3. Dinner (6 p.m.)	*Khichuri*	"	"	"
4. In between meals (10 a.m. & 3 p.m.)	Cow's milk	0.38	2.8	0.77
	Banana	0.29	0.20	1.24

TABLE 2

Physical, Clinical and Laboratory Data of Study Patients

	Cholera	Rotavirus
Number	19	13
Age (months)	43.2 ± 17.8[a]	23.2 ± 14.2[b]
Admission body wt. (kg)	9.3 ± 2.0	8.7 ± 1.8
Recovery body wt. (kg)	10.5 ± 2.5	9.1 ± 1.9
Acute stage 24 hour stool weight (g)	1700 ± 1500[c]	600 ± 300[d]
Recovery stage 24 hour stool weight (g)	162.0 ± 7.6	233.0 ± 152.4
Acute stage intestinal transit time (hr)	6.1 ± 3.5	6.2 ± 4.0
Recovery stage intestinal transit time (hr)	15.2 ± 10.4	12.3 ± 7.0
On admission		
Hct (%)	35.7 ± 3.9	31.2 ± 3.8
Serum Sp. gravity	1.028 ±0.003	1.027 ± 0.003
Serum Na^+ (mmoles/L)	132.6 ± 4.8[c]	138.3 ± 7.5[d]
Serum K^+ (mmoles/L)	3.8 ± 0.7	3.9 ± 1.0
Serum HCO_3^- (mmoles/L)	16.5 ± 3.7[a]	13.0 ± 2.5[b]

Student's t test - a versus b : $P<0.01$

c versus d : $P<0.05$

Figures indicate mean ± 1SD

TABLE 3

Mean Nutrient Intakes (± 1SD) During Acute Diarrhea (A) and After Recovery (R)

	Nitrogen (g/kg/day)		Fat (g/kg/day)		Kcal/kg/day	
	A	R	A	R	A	R
Cholera	0.32 ± 0.18[a]	0.57 ± 0.29[b]	2.03 ± 1.02[c]	2.99 ± 1.11[d]	69.85 ± 36.5[e]	108.4 ± 39.6[f]
Rotavirus	0.25 ± 0.09	0.32 ± 0.13[g]	1.88 ± 0.7	2.09 ± 0.93[h]	63.36 ± 23.4	80.8 ± 32.06[i]

Paired t test; a versus b : P<0.001; c versus d : P<0.001; e versus f : P<0.001;

Student's t test; b versus g : P<0.01; d versus h : P<0.05; f versus i : P<0.05.

TABLE 4

Coefficient of Absorption (± 1 SD) of Nutrients During Acute Diarrhea (A) and After Recovery (R)

	Nitrogen		Fat		K. cal	
	A	R	A	R	A	R
Cholera	43.7 ± 30[a]	78.3 ± 11.5[b]	71.5 ± 20.0[c]	89.1 ± 9.5[d]	80.8 ± 3.9[e]	91.1 ± 4.6[f]
Rotavirus	45.3 ± 22.3[g]	68.5 ± 13.0[h]	42.3 ± 27.6[i]	79.0 ± 20.0[j]	55.4 ± 23.7[k]	84.4 ± 8.6[l]

Paired t tests: a versus b : P<0.001; c versus d : P<0.001; e versus f : P<0.001;
g versus h : P<0.005; i versus j : P<0.001; k versus l : P<0.001;
Student's t test: b versus h : P<0.05; d versus j : P>0.05; f versus l : P<0.01.

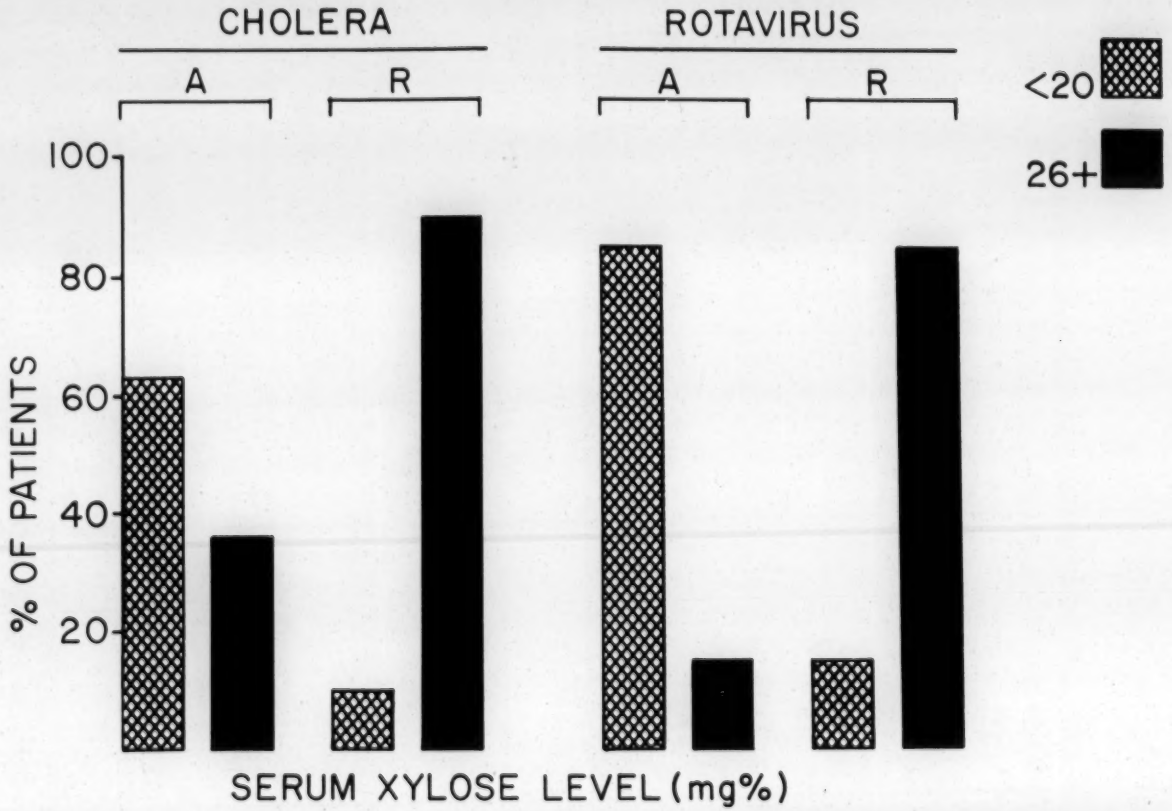

Figure 1
Xylose absorption in patients with cholera and rotavirus infection in both the acute stage (A) and after recovery (R).

DISCUSSION

The results presented here indicate that several differences exist with regard to the intake and absorption of nutrients during the acute and recovery stages of cholera and rotavirus infection.

In the acute stages of cholera the average intake of all of the nutrients (nitrogen, fat and calories) were reduced ($P<0.001$) by 37% compared to the recovery stage, whereas the mean intake of rotavirus patients was reduced by 18% ($P<0.005$). However, when the nutrient intakes during the recovery stages of cholera and rotavirus were compared, significant reductions ($P<0.05$) were seen in rotavirus patients. Cyclic-AMP mediated diarrhea in cholera is not associated with any mucosal lesion and the alteration of the mucosal enzymatic activity is transient (6). This is reflected in nearly complete absorption of nutrients in the recovery stage of cholera. In rotavirus infection, villous epithelial cells were specifically affected (17) and the inflammation possibly continues longer and is reflected by the prolonged malabsorption of nutrients. Furthermore, when net absorption of nitrogen, fat and calories was estimated in the acute stages of cholera and rotavirus, nitrogen absorption seemed to be affected equally, absorbing 44 and 45 percent respectively of the total ingested nitrogen. Rotavirus patients absorbed considerably less fat and calories in the acute stage than cholera patients. During the recovery stage cholera patients were absorbing significantly ($P<0.005$) higher percentages of nutrients than the rotavirus patients, suggesting more prolonged malabsorption in rotavirus than in cholera. However, from the present data (Table 2) it appears that the average body weight of cholera patients increased from 9.3 to 10.5 kg and rotavirus patients from 8.7 to 9.1 kg, indicating a return to positive nitrogen balance in the recovery stage.

An attempt was made to correlate the absorption of fat, nitrogen and calories with xylose absorption in both the acute and recovery stage. Although during the recovery period the rotavirus patients had prolonged period of malabsorption compared to cholera patients, the d-xylose test was not found to be affected.

Early feeding of these diarrheal patients did not seem to make the diarrhea worse. This was evident from a gradual decrease of stool volume during the acute stage of the study. This study did not address the mechanism of lower nutrient absorption in rotavirus compared to cholera, but known pathological changes in the small intestine caused by rotavirus infection may be responsible for this difference.

Although reduction of nutrient intake and malabsorption is associated with diarrheal illness, normal intake of food should be encouraged because substantial absorption does take place even during the acute stage of cholera and rotavirus. This might help in preventing further development of malnutrition. This observation has profound implications, especially in developing countries where cholera and rotavirus diarrhea are common.

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MAMMARY DYSPLASIA: ENDOCRINE PARAMETERS AND TOCOPHEROL THERAPY

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ABSTRACT

The effect of alpha tocopherol supplementation on serum hormones was studies in women with clinically confirmed mammary dysplasia and age-matched controls. Serum samples collected at monthly intervals on the 21st day of the menstrual cycle for a period of four months were analyzed for estradiol, progesterone, testosterone and dehydroepiandrosterone by radioimmunoassay. Eighty-five percent of the patients showed objective and subjective remission from the disease. Alpha tocopherol supplementation reduced the serum dehydroepiandrosterone levels of patients without affecting the concentrations of other hormones.

KEY WORDS: Mammary dysplasia, tocopherol therapy, hormone changes.

INTRODUCTION

Physicians have used vitamin supplements for treating fibrocystic disease of the breast with varying success (1). We have shown (2) that alpha tocopherol supplementation resulted in clinical improvement of fibrocystic disease of the breast; we have also correlated improvement with alterations in urinary steroid excretion. Since other workers (3-5) have shown abnormalities in steroid concentrations in these patients, this study was undertaken to determine the effects of alpha tocopherol supplementation on serum levels of different hormones in patients and age-matched controls. Results obtained with a smaller group of subjects were reported earlier (6).

MATERIALS AND METHODS

Twenty-six patients with fibrocystic disease of the breast, confirmed by biopsy and mammography, and 8 controls between 25 and 40 years of age were followed in this study after obtaining appropriate informed consent. All subjects had regular menstrual cycles, and no underlying medical or endocrine diseases. Subjects were placed on the placebo for 4 weeks followed by 600 IU/day of d,l alpha tocopherol acetate for a period of 8 weeks. Placebo and tocopherol were supplied by Hoffman La Roche, Inc. The tablets were taken orally in the mornings, with study subjects serving as their own controls.

Physical examinations were performed on day 21 of the menstrual cycle between 8:30-9:30 a.m., at which time a sample of blood was drawn. The serum was separated and separate aliquots were stored at -20°C until analysis. Clinical improvement was graded by two physicians, using previously described criteria. (2). Total disappearance of cystic lesions and tenderness was considered a good response, while a decrease in the number and size of the lesions with or without relief from pain was considered a fair response.

Serum tocopherol concentrations were measured by the method of Bieri et al. (7) Serum estradiol, progesterone, and dehydroepi androsterone (DHEA) were determined by radioimmunoassay techniques. Commercial kits were obtained as follows: Estradiol (Diagnostics Biochem. International, Tampa, Florida), Progesterone (Pantex, Santa Monica, California), DHEA (Wien Laboratories, Succasunna, New Jersey). Assays were performed according to procedures described in the kit with additional high and low control specimens included in each assay. Frozen aliquots of serum were used for each assay. All samples were assayed under identical conditions. The assay kits are routinely used in this laboratory to provide service to the hospital patients and low and high level controls are always included in the assays. The intra- and interassay errors for the different assays were either lower or similar to that reported in the protocols accompanying each kit. Alpha tocopherol did not cross react with any of the hormones measured.

Statistical analysis was performed using the Student's t-test for paired data with differences significant at $P<.05$.

RESULTS

Results in all tables are expressed as mean ± standard error of mean. Patients and controls showed increased serum concentration of alpha tocopherol following therapy (Table 1) indicating compliance and absorption. Of the 26 patients, 10 had "good" clinical response; 12 "fair"; and 4 were non-responders. The overall response rate was 85%.

TABLE 1

Levels and Changes in Serum Alpha Tocopherol (mg/dl)

	Before Therapy	After Therapy
Controls (n=8)	1.62 ±0.203	2.60±0.390*
Patients (n=25)	1.37 ±0.105	2.66±0.205***
Responders (n=21)	1.46 ±0.11	2.77±0.23***
Non-Responders (n=4)	0.898±0.174	2.04±0.261**

*P<0.02 **P<0.02 ***P<0.001

Table 2 shows that all patients are ovulatory as demonstrated by serum progesterone concentrations higher than 4 ng/ml in both patient and control groups. Alpha tocopherol supplementation did not significantly change the progesterone or estradiol (Table 3) concentrations.

TABLE 2

Serum Progesterone Concentrations (ng/ml) in Controls and Mammary Dysplasia Patients

	Before Therapy	After Therapy
Controls (n=8)	14.64± 3.33	16.66±4.13
Patients (n=22)	15.90± 2.26	17.27±2.12
Responders (n=19)	14.97± 2.12	16.34±2.25
Non-Responders (n=3)	23.70±10.06	23.17±6.09

TABLE 3

Serum Estradiol Concentrations (ng/ml) in Controls and Mammary Dysplasia Patients

	Before Therapy	After Therapy
Controls (n=8)	108.90±32.63	106.86±30.13
Patients (n=26)	91.91± 9.04	108.00± 9.28
Responders (n=22)	89.8 ±10.77	106.36±10.48
Non-Responders (n=4)	101.25± 7.47	117.00±19.99

Prior to therapy, DHEA concentrations (Table 4) in responders are signficiantly higher than in the corresponding controls, the difference between patients and controls disappearing after alpha tocopherol supplementation; however, non-responders also show a decrease in DHEA concentration as a result of supplementation.

TABLE 4

Serum DHEA Concentrations (ng/ml) in Controls and Mammary Dysplasia Patients

	Before Therapy	After Therapy
Controls (n=6)	5.08±0.08	5.08±0.11
Patients (n=18)	7.80±0.27	6.08±0.12
Responders (n=16)	8.13±1.4***	6.58±1.3
Non-Responders (n=2)	5.14±0.19	2.06±0.03***

***P<0.05 When responders are compared with controls before therapy and when DHEA levels in non-responders before and after therapy are compared.

DISCUSSION

A clinical response rate of 85% was observed similar to our earlier findings (2,8). It is important to note that we were administering pharmacologic quantities of tocopherol, approximately 20 times the recommended dietary allowance. We are presently carrying out a dose-response study to determine whether alpha tocopherol supplementation at 150 IU/day would be equally effective. In this study as well as in prior investigations, we monitored the laboratory status of the patients by hemograms, serum chemistries, etc. and noted no significant effects of the therapy on these parameters. In addition, other than changes in the breast examinations and breast symptoms in the patient group, no untoward effects of the therapy were noted. Naito et al. (9), who studied the effects of 600 IU of alpha tocopherol in men and women also showed no untoward effects. However, at least one investigator (10) has reported various side effects following alpha tocopherol intake. While we recognize the risks involved in ingesting more than a gram of alpha tocopherol in addition to different vitamins and minerals in an uncontrolled fashion, our results suggest that vitamin E supplementation at 600 IU/day is an effective and safe mode of treatment for patients with fibrocystic disease of the breast.

Although exhaustive investigations have been performed attempting to define the endocrine pathophysiology of patients with fibrocystic disease of the breast, no specific hormonal derangement can be implicated. Luteal phase progesterone concentrations have been reported to be lower in patients with mammary dysplasia when compared to controls (3); our study does not confirm such luteal phase dysfunction. We could not also confirm the abnormality in ratio between progesterone and estradiol reported by those investigators (3). Estradiol concentrations were similar in our patient and control groups, and we were not able to confirm England et al.'s (5) observation of elevated luteal phase estradiol in patients with fibrocystic disease of the breast.

Prior to therapy, the responding patients had significantly elevated serum concentrations of DHEA when compared to controls although the non-responders had similar levels. None of these patients were masculinized, or had stigmata of adrenal hyperfunction. After therapy the serum DHEA was decreased significantly in non-responders with a decreasing trend in responders. It is not possible to relate the alterations in the concentration of DHEA with the observed clinical improvement since inspite of a significant reduction in DHEA, the non-responders did not respond to therapy. From the present data we suggest that alpha tocopherol supplementation may be an inexpensive and safe way of treating patients with fibrocystic disease of the breast with no side effects and no significant changes in the levels of some hormones.

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REPRODUCIBILITY AND ACCURACY OF A FOOD FREQUENCY QUESTIONNAIRE USED FOR DIET INTERVENTION STUDIES

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ABSTRACT

The purpose of the present investigation was to determine whether altering the format of a food frequency questionnaire affected its accuracy and reproducibility. Three questionnaires were tested at one-month intervals. Questionnaire I contained 39 food items listed according to food categories. Questionnaire II contained 55 food items, presented according to the meal in which they were consumed. Questionnaire III contained 31 food items presented in a similar order to that in Questionnaire II. To determine the accuracy of the food frequency questionnaire, results were compared to those obtained from a simultaneously administered seven-day diet diary. Results from this study showed that food frequency questionnaires containing broad categories of food items presented in order of the meals in which they are consumed are the most reproducible. Highly reproducible results also can be obtained from seven-day diet diary, if data are calculated in terms of nutrients (i.e. carbohydrates, fats, proteins, etc.) and not specific food items. Finally, this study suggests that individuals more closely maintain their intake of nutrients, rather than specific food items.

Key words: reproducibility, Food Frequency Questionnaire, Diet Diary, Diet Intervention

INTRODUCTION

The best experimental procedure for determining dietary intake of individuals is a controversial issue. Balance studies which involve weighing all food eaten is the most accurate method currently available (1). However, this method is not only costly and time-consuming, but cannot be carried out in most laboratories. Therefore, questionnaire-type techniques for establishing food intake were developed (2-11). Although these surveys are relatively inexpensive and easy to administer, it is questionable whether the information derived from them is accurate and reproducible.

A commonly used questionnaire method is the diet diary (3). In this method, a subject records all foods eaten, preferably at the time of consumption. Trulson and McCann (3) reported that one week dietary recall methods only reflected a person's consumption during that week rather than their characteristic patterns of food consumption. Conversely, diet history or food frequency techniques in which subjects report the number of times they consume a particular food item during a designated period (i.e. 1 month) appears to be a reliable or reproducable method of determining nutrient intake (5). However, the accuracy of this method is difficult to determine. In addition, it is not known whether the reliability of food frequency data is significantly affected by the number of food items presented and the order in which they are presented.

The purpose of the present study was to determine (a) whether the length of the food frequency questionnaire affected its reproducibility; (b) to determine the reproducibility of data derived from the diet diary technique; and (c) to determine the accuracy of the diet diary in predicting nutrients, as determined by the Food Frequency Technique.

METHODS

The present study was designed to determine the most reproducible and accurate format for the Food Frequency Questionnaire that can be used in a dietary intervention study with a small number of participants (i.e. <20). Three types of food frequency questionnaires were studied.

Questionnaires

In Experiment 1, the food frequency questionnaire consisted of 39 items listed according to the following food categories: dairy, hot beverages, meats, carbohydrates, fruits, vegetables, desserts and miscellaneous foods. The exact order of presentation is in Table 1.

Fifty-five food items grouped according to the meal in which the food might be consumed were utilized in Experiment 2. Table 2 contains the exact order of presentation of food items from Questionnaire 2 (long survey).

Food items in the third experiment were presented in a similar order as in Experiment 2. However, foods were classified into broader categories, resulting in the 31 food items indicated in Table 3 (short survey).

In Experiments 1-3, participants were asked to place the number from the code list (Table 4) that best indicated how often they included the foods listed in their present day diet.

In the final experiment (Experiment 4), participants from experiment 1 were asked to record in a seven day diet diary the amount and type of all foods consumed at the time of consumption. After recording food consumption for one week, participants were asked to complete the Food Frequency Questionnaire utilized in Experiment 1 (phase 1, experiment 1). Four weeks later, all participants were asked to complete a second 7-day diet diary.

Subjects

Subjects in Experiments 1 and 4 were involved in an ongoing study in our laboratory. Participants in Experiments 2 and 3 were selected specifically for these experiments. Participants were over 21 years of age, living in the New York metropolitan area, and randomly selected from a subgroup of members of the American Health Foundation and their families. All subjects signed consent forms and knew that they would be asked to complete questionnaires relating to food intake at least at 2 time intervals.

The sex distribution for each experiment was as follows: Experiment 1, 5 males, 7 females; Experiment 2, 1 male, 11 females; Experiment 3, 5 males, 7 females; Experiment 4, 5 males, 7 females.

Analytical Approach

To examine the reproducibility of data obtained from various types of food frequency questionnaires utilized in Experiments 1-3, surveys were administered twice at 4 week intervals (test periods 1 and 2, respectively). Data coded for computer analysis by trained personnel utilizing the results obtained directly from the Food Frequency Questionnaire were termed initial data. Grouped frequencies were obtained by recoding initial data into 3 categories; rarely or never, 1-7 times a week and more than 7 times a week.

Information from Experiment 4 was transferred to separate coding sheets in a manner similar to that in Experiments 1 - 3. Information from the diet diaries was computed into macro and micro nutrients found in Table 5, utilizing the USDA Handbook #8. Statistical significance was determined by a Student's t-test.

To determine the accuracy of the Food Frequency Surveys, participants in Experiment 1 were asked to record all food items eaten at the time of consumption in a 7-day diet diary. This data was recorded by trained coders onto the Food Frequency Questionnaire utilized in Experiment 1. Codes used are in Table 4. Results were compared using a Spearman's Rank Order Correlation Coefficient to the results obtained from the Food Frequency Questionnaire utilized in Experiment 1.

RESULTS

Results from Experiment 1[a] are shown in Table 1. Nineteen of the 39 food items tested were significantly correlated when foods were presented according to food type (i.e. meat, dairy, desserts, etc.) (Table 1). When the frequencies were grouped in Experiment 1[b], 67% of food items with sufficient data to compute a Spearman's Correlation Coefficient were significantly correlated. A sex difference in the reproducibility of the percentage of food types consumed is demonstrated in Table 1. Eight of 28 (29%) and 13 of 34 (38%) of the food items were consumed with similar frequencies for male and female participants, respectively.

TABLE 1

Spearman's Rank Order Correlation Coefficient Between The Various Dietary Methods Used In Experiments 1 and 4

	Experiment 1[a] Initial Freq. N=12	Experiment 1[b] Grouped Freqs. N=12	Experiment 1[c] Grouped Freq. Males N=5	Experiment 1[c] Grouped Freq. Females N=7	Experiment 4[d] Initial Freq. N=12
Whole milk	.54*	.81***	e	.97**	.34
Skimmed milk	.66**	.68**	1.00***	.53	e
Cottage cheese	.74	.63	.61	.73*	.91***
Other cheese	.63**	.53*	.61	.47	.38
Ice cream	.48*	.56*	.61	.65	.19
Butter	.91***	.94***	1.00***	.90**	.57*
Margerine	.52*	.44	.25	.47	.43
Eggs	.49*	.16	e	.09	.19
Reg. Brewed Cof.	.77**	.77**	.83*	.73*	.21
Instant Coffee	.38	.23	.00	.54	.39
Decaf. Coffee	.44	.67**	e	.65	e
Tea	.42	.65**	.61	.59	.50*
Hamburger	.31	.48*	.61	.55	.57*
Beef/steak	.36	.48*	.61	.42	.58*
Veal/lamb	.50*	e	e	e	.14
Poultry	.00	e	e	e	.43
Pork	.62*	.54*	e	.47	.41
Ham/Sausage	.42	.16	e	.65	.83***
Fish	.82***	.85***	1.00***	.73*	.46
Cereals	.89***	1.00***	1.00***	1.00***	.83***
Rice	.34	1.00***	1.00***	1.00***	.06
Pasta	.48*	.48*	.61	.42	.13
Potatoes	.81***	.82***	1.00***	.73*	.26
Corn	-.10	.41	e	.17	.35
Bread - white	.34	.50*	.50	.58	.85***
Bread - whole wheat	.59*	.53*	.91*	.24	-.01
Fresh citrus fruit	-.01	.49*	.25	.73*	.32
Fresh fruit	.44	.68**	.67	.65	.38
Citrus fruit juice	.47	.26	e	.26	.33
Green leafy vegetables	.64**	e	e	e	.71**

TABLE 1 (continued)

Spearman's Rank Order Correlation Coefficient Between The Various Dietary Methods Used In Experiments 1 and 4

	Exper. 1[a]	Exper. 1[b]	Exper. 1[c]		Exper. 4[d]
Fresh vegetables	.29	e	e	e	.03
Canned vegetables	.35	.10	-.17	.35	.00
Beans/lentils/peas	.10	.00	.61	-.40	.31
Candy	.63**	.25	.61	.09	.54*
Cakes	.87***	1.00***	1.00***	1.00***	.37
Sugar	.44	.39	-.41	.82**	.43
Nuts	.37	.48*	-.41	1.00***	.34
Pickled foods	.77**	1.00***	e	1.00***	-.04
Smoked foods	.38	e	e	e	e
	19/39 or 48%	23/34 or 67%	8/28 or 29%	13/34 or 38%	10/36 or 28%

[a] Experiment I compared values derived from food frequency questionnaire given at 4 wk intervals

[b] Experiment I[b] compared values derived from grouping frequencies on the food frequency questionnaire

[c] Experiment I[c] compared values derived from grouping frequencies on the food frequency questionnaire coparing male and female responses

[d] Experiment IV compared values derived from a food frequency questionnaire and a 7-day dietary recall method given simultaneously

[e] Insufficient data to compute Spearman's Correlation Coefficient

* $P<0.05$

** $P<0.01$

*** $P<0.001$

TABLE 2

Spearman's Rank Order Correlation Coefficient - Elongated Food Frequency Questionnaire [a,b]

	Initial Freq. N=12	Grouped Freq. N=12
Rolls	.80***	.83***
Sweet rolls	.71**	.60*
Corn muffins	.54*	-.12
White bread	.31	e
Whole wheat bread	.68**	.60*
Pancakes	.92***	.64**
Cereal	.42	-.08
Orange/grapefruit juice	.70**	.85***
Other juices	.71**	.57*
Oranges/grapefruit	.74**	.73**
Other fresh fruit	.74**	.89***
Canned fruit	.88***	.85***
Eggs	.41	.41
Bacon	.76***	.64**
Soups	.66**	.68**
Green leafy salads	.19	.54*
Hamburger	.81***	.48*
Hot dogs	.60*	.55*
Meat sandwiches	.30	e
Poultry sandwiches	.71**	.27
Fish sandwiches	.33	.03
Cold cuts(e.g. ham, bologna)	.82***	.82***
Non-meat(e.g. cheese, eggs)	.41	.32
Salads	.64**	.72**
Cottage cheese	.16	.32
Other cheese	.74**	.82***
Chinese food	.68**	.68**
Italian pasta	.61**	.23
Pizza	.19	.18
Whole milk	.32	e
Low fat milk	.85***	.88**
Beef	.65**	.63**
Fish	.87***	.76***
Pork	.87***	.84***
Poultry	.30	.27
Fresh vegetables	-.08	.27
Frozen vegetables	.39	.52*
Potatoes	.74**	.59*
Rice	.79***	.75**
Cakes and pies	.37	.05
Cookies	.52*	.50*
Candy	.32	.28
Ice cream	.79***	.69**
Snacks	.66**	.69**
Nuts	.48	.62**
Diet soda	.65**	.43
Regular soda	.73**	.67**

TABLE 2 (continued)

Spearman's Rank Order Correlation Coefficient - Elongated Food Frequency Questionnaire [a,b]

	Initial Freq. N=12	Grouped Freq. N=12
Coffee	.31	.26
Regular brewed coffee	.93***	.90***
Decarfinated coffee	.84***	1.00***
Tea	.58*	.59*
Hot chocolate	.75**	.43
Beer	.53*	.53*
Wine	.73**	.84***
Other Alcoholic Beverage	.78***	.70**
	38/55 or 69%	36/55 or 65%

[a]
* P< .05
** P< .01
*** P<.001

[b] unable to correlate by sexes; males = 1, females = 11

When data from a Food Frequency Questionnaire and a dietary diary given simultaneously were compared, 28% of the food items with sufficient data to compute were consumed with the same frequencies between the two test periods (Table 1).

Table 2 shows that when 55 food items were grouped according to the meal in which they were consumed, 69% of the initial frequencies were similar between the two test periods. Sixty-five percent of all food items analyzed according to group frequencies were the same between test periods. When the shortened Food Frequency Questionnaire was tested, 27 of the 31 food items presented were recalled with similar frequency in the initial group and 24 of the 29, or 83% of the grouped frequency food items, were similar (Table 3). The percentage of food items recalled with similar reproducibility utilizing the shortened Food Frequency Questionnaire was slightly higher for females (78%) than for males (67%) (Table 3).

The average daily consumption of 20 macro and micro nutrients derived from a 7-day diet diary is found in Table 4. The amount of all nutrients consumed, except vitamin A, was similar at the two test periods one month apart.

TABLE 3

Spearman's Rank Order Correlation Coefficient

Shortened Food Frequency Questionnaire[a]

	Initial Frequency N=15	Grouped Frequencies N=15	Males N=4	Females N=15
Cereal	.20	.68**	1.00***	b
Eggs	.80**	1.00***	1.00***	1.00***
Bread or rolls	.89***	.88***	1.00***	.83***
Juice	.38	.70**	1.00***	.57*
Pancakes	.70**	.49*	.00	.67**
Breakfast meats	.83***	b	b	b
Meat Sandwich	.81***	1.00***	1.00***	1.00***
Non-meat sandwich	.75***	.76***	b	.77**
Fresh fruits	.25	-.19	b	.24
Canned fruits	.71**	.53*	.50	.50
Fresh vegetables	.68**	.42	b	.67**
Canned vegetables	.58**	.28	b	.38
Cottage cheese	.49**	.36	b	.40
Beef	.78***	.99***	1.00***	.98***
Poultry	.84***	.76***	1.00***	.69**
Fish	.83***	1.00***	b	1.00***
Chinese food	.59**	.49*	b	.45
Italian food	.53**	b	b	b
Potatoes	.85***	.87***	1.00***	.83***
Rice	.36	.34	-.33	.44
Soup	.66**	.66**	1.00***	.56*
Cakes	.69**	.50*	.33	.57*
Ice Cream	.85***	.82***	.58	1.00***
Candy	.84***	.82***	1.00***	.77**
Nuts	.80***	.46*	-.33	1.00***
Coffee	.81***	1.00***	1.00***	1.00***
Tea	.73***	.73***	.82	.68**
Milk	.71***	.49*	b	.57*
Soda	.83***	.89***	1.00***	.81***
Beer/wines	.87***	.87***	1.00***	.84***
Snacks	.70**	.85***	1.00***	.81***
	27/31 87%	24/29 83%	14/21 67%	22/28 78%

a

* P< .05
** P< .01
*** P< .001

b Insufficient data to compute Spearman's Correlation Coefficient.

TABLE 4

Code List

1 - Rarely or never
2 - Infrequently (1-3 times/month)
3 - Moderately (1-3 times/week)
4 - Daily (4-7 times/week)
5 - More than 7 times/week

TABLE 5

Mean Nutrient Consumption of Foods Consumed According to the Seven Day Dietary Diary[1,2]

	Test Period I	Test Period II
Calories	2036.8± 74.2	2182.9± 127.5
Protein (g)	70.3± 4.2	76.6± 6.2
Fat (g)	80.5± 5.0	83.1± 7.6
Carbohydrate (g)	256.6± 9.5	282.5± 14.0
Fiber (g)	6.0± 0.3	6.4± 0.6
Ash (g)	15.3± 0.9	14.1± 0.5
Calcium (mg)	555.2± 51.0	466.8± 21.2
Phosphorus (mg)	1041.9± 83.9	955.1± 34.9
Iron (mg)	13.2± 1.4	13.4± 1.6
Sodium (mg)	3277.5±272.2	3192.0± 203.0
Potassium (mg)	2183.4±164.0	2000.0± 95.2
Vitamin A (IU)	4674.8±543.7*	6255.8±1715.3
Thiamin (mg)	1.1± 0.1	1.0± 0.1
Riboflavin (mg)	1.4± 0.1	1.2± 0.1
Niacin (mg)	18.3± 1.4	17.8± 0.8
Ascorbic acid (mg)	92.3± 19.1	129.7± 3.9
Saturated fatty acid (g)	26.1± 1.9	25.7± 2.0
Oleic acid (g)	27.1± 2.0	27.2± 1.9
Linoleic acid (g)	21.2± 6.7	13.8± 1.1
Cholesterol (mg)	295.8± 30.5	311.0± 28.1

1 Each value is the mean ± the standard error on the mean derived from 12 seven-day diet diaries.

2 Values with an asterisk denote that statistically different ($P<0.05$) amounts of that nutrient were consumed at test period 1 and test period 2, which were 4 weeks apart.

DISCUSSION

Both food frequency and dietary recall techniques are often used as standard experimental procedure in dietary intervention studies. The reliability and accuracy of these experimental methods has been questioned by numerous investigators (3,4,6,11). Results from this study show that the degree of accuracy and reproducibility of the food frequency technique is dependent on a complex array of factors, including the sex of the participants, the number of food items, and the order in which they are presented. The apparent differences in reproducibility can be explained by inherent differences in food diaries which are prospective in nature, and food frequency questionnaires, which are retrospective.

Results from the seven-day diet diary were interpreted in two fashions. Utilizing data from the USDA Handbook #8, various macronutrients and micronutrients were determined by computor analysis. Of the 20 nutrients examined, vitamin A was the only nutrient where significantly different levels were consumed at 1 month intervals. However, similar quantities of the majority of nutrients which tend to be of epidemiological interest in terms of many chronic diseases, such as heart disease and cancer, were similar between the two test periods. If information is needed on consumption of vitamin A which did not correlate utilizing the diet diary technique, other methods for determining data on the consumption of that particular nutrient would have to be designed.

The second method for analyzing data obtained from the seven-day diet diary was through recoding data onto a Food Frequency Questionnaire. These results were compared to those obtained from a simultaneously completed Food Frequency Questionnaire which was similar to the one used for recoding. Only 10 of the 39 food items examined between the two survey techniques given at the same time were significantly correlated. These results have been supported by others. (4,6).

The lack of correlation between products such as hot dogs, fish and canned fruit, etc. consumed as determined by the Food Frequency Questionnaire and the seven-day diet diary challenges the accuracy of either method in correctly establishing food intake. However, since the seven-day diet diary was shown to be highly reproducible (Table 5) in conjunction with the fact that the participants in our study were highly educated, we feel that this method is reasonably accurate. Conversely, only 48% of the food items consumed, as determined by Food Frequency Questionnaire I were significantly correlated between the two test periods (Table 1). These results suggest that foods from which individuals derive their nutrients significantly change even during a short period of one month. Clark *et al.* (12) reported that a one-week diet diary shows a mean consumption of foods for a period of one week and not necessarily a characteristic food consumption pattern. According to our data, consumption of specific food products may change on a weekly basis, while consumption of particular nutrients tends to remain the same.

The observation that individuals more closely maintain the intake of specific nutrients than specific foods is supported by additional food frequency data (Tables 2,3). When the Elongated Food Frequency Questionnaires were administered at one month intervals, 69% (38/55) food products studied were significantly correlated. Twenty-seven of the 31 (87%) of the food products examined were significantly correlated utilizing the shortened Food Frequency Questionnaire. Therefore, as the Food Frequency Questionnaire became less specific, the reproducibility of the questionnaire increased. This suggests that individuals tend to eat from broad categories of foods (i.e. beef) vs. more specific food items (i.e. hamburgers). This observation is highly probable in light of the growing trend to purchase the "weekly special" at the supermarket, rather than purchasing the particular food item desired, regardless of the price.

Females participating in the food frequency studies tended to provide more reproducible results than male participants (Table 1,3). This may reflect the role of the female in purchasing and preparing the majority of meals for the family. However, the percent differences between males and females were small (approximately 10%) and should not present a major problem in analyzing food frequency data.

Grouping frequencies into three categories vs. five had almost no effect on the reproducibility of the data obtained from the Food Frequency Questionnaire when the food items were presented according to the meal in which the food might be consumed. A similar trend was observed regardless of the number of food items in the questionnaire (Tables 2,3). Conversely, a 20% increase in the number of food items consumed with similar frequencies was seen when food items were presented according to food categories (Table 1). This demonstrates that altering one parameter in a Food Frequency Questionnaire, such as order of presentation, may have a significant effect on a second parameter, such as the reproducibility of grouped and ungrouped food frequency data.

Beaton et al. (11) observed that intra-individual variation may bias estimates of correlation coefficients which reflect a large day to day variation in the composition of self-selected diets. Therefore, Beaton et al. (11) question the precision in estimating an individual's usual intake from a 24-hour dietary recall. Consequently, we chose to administer a 1-week diet diary. However, there are still some important limitations to the present study. Primarily, we are assuming that the seven-day diet diary is an accurate record of food consumption against which we can compare the accuracy of data obtained from a Food Frequency Questionnaire. We are aware of this problem and feel there is no feasible way to avoid this assumption. In addition, our test group was relatively small and participants belonged to a biased population. We intentionally chose a small sample size, since we hoped to design a Food Frequency Questionnaire that could be used in diet intervention studies and not epidemiologic studies.

With these factors in mind, the following conclusions can be drawn:

1. Food Frequency Questionnaires administered at one month intervals containing broad categories of food items presented in order of the meals in which they are consumed provide the most reproducible results.

2. When specific nutrients from a seven-day diet diary given at one month periods are calculated, highly reproducible results are obtained.

3. The lack of correlation between dietary data obtained from a Food Frequency Questionnaire and data obtained from a diet diary suggests that individuals more closely maintain their intake of specific nutrients rather than their intake of specific food items.

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COMPARISON OF SEVEN-DAY DIET RECORD WITH MEASURED FOOD INTAKE OF TWENTY-FOUR SUBJECTS

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ABSTRACT

A comparison was made between calorie intakes recorded for 7 days by 12 men and 12 women and the calorie levels they consumed during an 18-week controlled dietary study during which body weight was maintained. A large discrepancy was found in the calories consumed during the controlled study and the calories recorded on the 7-day record. The difference between average daily kilocalories women consumed during the study and the number on the diet record was more than 900. Men reported consuming about 500 kcal less per day than they consumed to maintain weight. Men and women both consumed about 43% carbohydrate, 16% protein and 41% fat. P/S ratio was about 0.4 and cholesterol intake was relatively low, averaging 415 mg in men and 335 in women. On the average, men met the RDA for all nutrients reported, but women had low intakes of iron, calcium, and vitamin A. The average number of daily meals consumed was 3.42. The subjects consumed an average of 16% of the calories between midnight and 11:00 AM, 32% between 11:00 AM and 4:00 PM, and 52% between 4:00 PM and midnight. These results indicate that there may be a wide range of calorie intakes at which subjects can maintain body weight. If caloric intakes recorded by the women are accurate and representative, they may have difficulty in obtaining necessary amounts of calcium, vitamin A, and iron without dietary changes or vitamin and mineral supplementation.

Dietary records Recall Food intake

INTRODUCTION

The reliability of diet records, diet histories, and 24-hour recalls has been debated among nutritionists for many years (1-3). The amounts of food available for consumption or disappearance data (4) have been used to estimate average nutrient intakes (5). In the past decade the amount of food available for consumption per capita has increased (4) while the number of calories reported to be eaten by individuals has decreased (6). There seems to be a wide disparity in the amount of food that disappears and the amount of food that individuals report they consume. The new Recommended Dietary Allowances (RDA) for calories reflect an apparent reduction in average intakes (7), but recognize the wide individual differences in caloric needs by including an 800-kcal span in recommendations. It is difficult to know whether discrepancies in recorded intakes reflect inaccurate reporting by the patient,

incomplete or erroneous nutriture data, differences in thermogenesis, or actual low caloric intake. Generally, in surveys of nutritional intakes only averages for various age and sex groups are reported; however, in individual clinical cases, specific numbers of calories required by individuals to maintain body weight are needed. Therefore, if the problems with reliability can be resolved, diet records could be an important tool in determining calorie levels and types of foods consumed by certain patients.

Eating patterns reportedly are changing in this country. More meals are consumed away from home (8). Many people commute long distances to work, skip breakfast often and eat a light lunch and, therefore, consume most of their calories in the evening (9). Frequency of eating is reported to be inversely proportional to body fat (10). Gorging or consuming few meals may adversely affect insulin response (11). Therefore, the eating patterns of subjects while on self-selected diets are of interest.

The purpose of this paper was to compare eating patterns and nutrient intakes to recommended daily allowances and to compare intake levels with controlled levels which are known to maintain body weight. The amounts of calories fed to 12 men and 12 women during a controlled study were compared to the amounts reported eaten by them before the study. Throughout the 18 weeks of the study, all food was measured and body weights were recorded daily and maintained. Eating patterns and the amounts of various nutrients consumed were determined from 7-day diet records. In this paper, these intakes will be compared with previously published intake data (1-3).

MATERIALS AND METHODS

Twelve men and 12 women selected for the study ranged in age from 21 to 52 years. Before the controlled feeding period, they were asked to keep a 7-day dietary record. For each food, they recorded the time, place, amount, brand name (if applicable) and method of preparation. A dietician gave subjects detailed written and oral instructions for determining amounts of foods and recipes for mixtures such as casseroles or baked goods. Subjects were asked to carry food record sheets with them and record foods at the time they were eaten. Salad dressings, gravy, cream, sugar and condiments were recorded. Subjects were asked to eat normally during the record period. No subject was on a weight-loss diet during this period. Amounts of nutrients were calculated by use of data from Agriculture Handbooks No. 8 (12) and No. 456 (13) and information from various manufacturers (14-18).

During the study, the subjects were fed for 18 weeks in a modified gorging pattern: 25% of the calories at breakfast and 75% at dinner. Various levels of sucrose were fed during the 18-week period, some of the results of which have previously been reported (19). The gorging pattern was selected to emphasize the effects of sucrose. The 18-week study was divided into three 6-week periods. During the week, all meals were eaten under supervision in the Human Study Facility Dining Room. Subjects were requested to eat all food served. Food for the weekends was packed in styrofoam containers and taken home by subjects with instructions for time of meals and methods for heating meals. Subjects had neither gained nor lost more than 15 pounds in the previous year. Body weights taken at the time of selection and collection of food records agreed well with initial body weights at the onset of the experimental period 2 to 3 months later. Initially, subjects were fed 38 kcal/kg of body weight. Calorie levels were adjusted in 200-kcal intervals to maintain initial body weight. All foods were weighed to within 0.5 g. Subjects were requested to maintain initial activity levels during the

experimental period. Activity sheets were filled out daily by each subject. The body weight and caloric intake data were analyzed by a split plot analysis of variance. Differences were determined by Duncan's multiple range test (20). Seven-day record data for men and women were compared with a Student's t test.

RESULTS

Body weights, initial and averages during the three 6-week experimental periods, appear in Table 1. Men weighed significantly more than women initially and during the study, $P < 0.02$ and $P < 0.03$, respectively. According to Metropolitan Life Insurance Company tables, the subjects averaged about 110% of ideal body weight, and four subjects exceeded 115%. Body weights increased slightly in Period III, but none of the weights for the three periods differed significantly from the pretest weights.

Calorie levels differed markedly between the 7-day dietary records and the experimental periods, especially for women (Table 2). Calorie levels for both men and women did not change during the three diet periods. Calorie intakes of both men and women were significantly higher during all three periods than intakes reported in the dietary record.

The percentages of food consumed during three time periods which encompassed breakfast, lunch, and dinner were determined (Table 3). Breakfast was from midnight to 11:00 AM, lunch from 11:00 AM to 4:00 PM, and dinner from 4:00 PM to midnight. According to the diet records men and women consumed almost exactly the same percentages of food during the three periods; 16 to 17% at breakfast, 32% at lunch, and 52% at dinner. The number of meals eaten per day was also determined. At least 100 kcal within 1 hour was considered to be a meal. The number of meals consumed was slightly higher for men (3.57) than for women (3.26), but the difference was not significant. The average percent RDA exceeded 100% for men and women for protein, thiamin, riboflavin, niacin, vitamin C, and phosphorus (Table 4). For men only, at least 100% of RDA was met for vitamin A, calcium, and iron. Vitamin A intakes of the women ranged from 232 RE to 1246 RE and 3 of the 12 women consumed less than 50% of the RDA. Calcium intakes varied widely from 149 to 1211 mg. Three of the 12 women had intakes of less than 50% RDA for calcium. Iron intakes ranged from 6.58 to 15.98 mg/day. No woman met the RDA for iron--intakes were below 50% for three and below 66% for seven.

Average percentages of calories from protein (16%), fat (41%), and carbohydrate (43%) on the 7-day diet records were similar for men and women (Table 5). P/S ratios of men and women were almost identical, 0.42 and 0.45, respectively. Men consumed considerably more fiber than women, 4.85 compared to 2.79 g. Cholesterol intake was somewhat lower in women than in men, but not significantly different.

DISCUSSION

The most disturbing result from this experiment is the huge discrepancy between the intakes of calories reported in the dietary record and the amounts fed to maintain weight during the 18-week experimental period. The dietary records do agree fairly well with data for 24-hour recall and 2-day records from the USDA Nationwide Food Consumption Survey, 1977-78 (21) which found men ages 35 to 50 years to consume an average of 2314 kcal and men 20 to 34 years

TABLE 1

Average Body Weights of Subjects Before Experiment and During Three 6-week Experimental Periods

Subject number	Age	Initial body weight	Period body weight* I	II	III
		kg		kg	
Men					
1	25	68.8	66.6	66.8	69.2
3	49	64.4	64.0	63.6	63.3
6	48	80.1	81.3	82.0	79.8
7	31	91.6	90.4	90.4	91.1
8	47	102.6	101.8	103.2	102.5
10	47	83.4	83.7	84.4	84.4
14	24	67.4	67.2	66.8	68.2
15	43	77.6	77.9	79.4	79.5
17	39	73.2	72.5	70.2	71.7
20	39	83.4	84.5	84.4	84.7
22	33	72.0	71.3	71.7	73.3
24	39	72.6	70.3	69.9	70.9
Mean†		78.1^{c,d}	77.6^{c}	77.7^{c,d}	78.2^{d}
Women					
2	44	71.0	72.4	72.9	72.6
4	39	55.6	54.4	53.9	54.7
5	21	74.8	75.4	74.3	74.4
9	36	75.2	76.9	76.0	75.5
11	49	69.4	68.4	67.5	67.8
12	51	67.0	66.4	66.6	67.1
13	38	76.6	77.2	78.6	77.6
16	21	70.2	70.4	71.1	72.7
18	26	42.0	41.5	41.5	42.0
19	21	55.0	55.3	55.6	55.7
21	24	74.4	73.9	74.3	75.6
23	43	73.8	75.5	75.7	75.9
Mean†		67.1^{e}	67.3^{e}	67.3^{e}	67.6^{e}

* Period weights were taken during weekdays. Period averages for individuals represent 30 weights.

† Means not sharing a common superscript letter are significantly different, $P < 0.05$, according to Duncan's multiple range test.

TABLE 2

Average Daily Caloric Intakes of Subjects According to 7-day Diet Record and During Three 6-week Experimental Periods

Subject number	7-day diet record average daily intake	6-week periods* average daily intake		
		I	II	III
	kcal		kcal	
Men				
1	3964	3167	3795	3900
3	2431	2500	2500	2500
6	1535	2784	2300	2100
7	3557	3586	3881	3900
8	2637	3900	3590	3500
10	2287	3100	3071	2833
14	1583	2543	2890	3100
15	3458	2900	2886	2633
17	2063	2700	3128	3686
20	1908	3300	3076	3038
22	2508	2848	2900	2686
24	2182	2879	3300	3686
Mean†	2509[d]	3017[e]	3110[e]	3130[e]
Women				
2	924	2700	2700	2700
4	1268	2100	2195	2300
5	1872	2733	2500	2500
9	2926	2900	2900	2900
11	1739	2700	2700	2700
12	1473	2500	2500	2500
13	1120	2900	2600	2500
16	1323	2700	2700	2467
18	1594	1657	2033	2100
19	1024	2262	2300	2300
21	1764	3205	3300	3300
23	2548	2700	2700	2700
Mean†	1631[c]	2588[d]	2594[d]	2581[d]

* Period intake levels represent an average of 42 daily intakes.

† Means not sharing a common superscript letter are significantly different, $P < 0.05$, according to Duncan's multiple range test.

TABLE 3

Percent of Daily Calories Consumed During Three Meal Periods and Average Daily Number of Meals According to 7-day Diet Record*

Subject number	Percent daily calories consumed midnight-11 am	11 am-4 pm	4 pm-midnight	Average daily number of meals†
Men				
1	11	34	55	3.86
3	7	44	49	4.00
6	33	27	40	3.00
7	18	35	47	3.86
8	21	25	54	3.86
10	14	40	46	3.57
14	0	25	75	1.71
15	12	14	73	3.43
17	37	43	20	3.86
20	20	30	50	3.71
22	9	32	59	4.57
24	16	26	58	3.43
Mean	16.5 ± 3.0	31.2 ± 2.5	52.2 ± 4.2	3.57 ± 0.20
Women				
2	12	29	59	2.50
4	15	46	39	4.00
5	24	41	35	3.57
9	21	39	40	4.71
11	20	45	35	3.71
12	24	19	56	3.43
13	9	38	53	2.43
16	6	20	74	2.43
18	31	17	52	2.34
19	5	21	74	2.86
21	23	33	44	3.00
23	9	27	64	3.32
Mean	16.6 ± 2.4	31.2 ± 3.0	52.1 ± 4.0	3.26 ± 0.21

* There were no significant differences between men and women in percent of calories consumed at meals and number of meals consumed.

† A meal was considered to be at least 100 calories within 1 hour.

TABLE 4

Average Daily Intakes of Various Nutrients Compared with RDA for 12 Men and 12 Women According to 7-day Diet Record

Nutrient	Men		Women	
	RDA*	daily average†	RDA*	daily average†
Vitamin A (RE)	1000	1854 ± 486	800	757 ± 104
Thiamin (mg)	1.4	1.74 ± 0.25	1.0	1.01 ± 0.11
Riboflavin (mg)	1.6	2.38 ± 0.34	1.2	1.39 ± 0.16
Niacin (mg)	18	28.8 ± 3.7	13	14.6 ± 1.2
Vitamin C (mg)	60	139 ± 16	60	66 ± 8
Calcium (mg)	800	897 ± 98	800	624 ± 87
Phosphorus (mg)	800	1431 ± 131	800	961 ± 94
Iron (mg)	10	18.0 ± 2.3	18	11.2 ± 0.8
Sodium (mg)	900-2700	3374 ± 248	900-2700	2620 ± 300

* 1980 Recommended Dietary Allowances for men and women aged 23 to 50 years, Food and Nutrition Board, National Academy of Sciences-National Research Council.

† Average of 12 subjects ± SEM.

TABLE 5

Average Daily Percent of Calories from Various Nutrients, and Cholesterol, Fiber Intakes, and P/S Ratio of 24 Subjects According to 7-day Diet Record*

	Men	Women
Percent calories carbohydrate	43.1 ± 2.3	43.0 ± 2.3
Percent calories fat	41.4 ± 1.5	40.6 ± 2.0
Percent calories protein	15.7 ± 1.0	16.4 ± 1.0
Cholesterol (mg)	415 ± 63	335 ± 45
Crude fiber (g)	4.85 ± 0.48	2.79 ± 0.20
P/S ratio	0.42 ± 0.06	0.45 ± 0.05

* All values mean ± SEM of 12 daily averages. There were no significant differences between men and women in parameters listed on this table.

of age to consume 2482, while women ages 35 to 50 years consumed 1514 kcal and women 19 to 34 years of age consumed 1617. Hanes intake data for subjects aged 35 to 44 years were also comparable (22). These values are somewhat less than data obtained in the 1965-6 USDA Household Survey (23).

In our sample, men recorded an average of 2509 kcal/day and the women, 1631 kcal/day. When the subjects were fed to maintain weight, average daily intakes were about 500 kcal more for men and over 900 kcal more for women than they reported eating. There are several possibilities for this discrepancy. Since the percent of calories from carbohydrate, fat, and protein agree fairly well with amounts expected from both recall data and food availability charts, it may be that the amounts of food are underestimated, but that all or most of the foods eaten are properly recorded. It may also be possible that the act of recording food intake causes intakes to be reduced and, although they may be accurate for that period, they are not typical of the individual's normal intake. It is also possible that many people can maintain their weight over a relatively wide range of caloric intake (24, 25). Activity levels could have changed between the dietary record period and the experimental periods, although subjects were asked to maintain pre-experiment activity levels, and cursory examination of daily activity sheets did not indicate any changes.

If recorded caloric intakes are accurate, our women subjects would have a serious problem in meeting RDA for various micronutrients without some dietary supplements. Recorded intakes of B vitamins are generally adequate, because many foods now are enriched with these vitamins.

Average calcium levels of the women were well below the RDA; only three consumed over 800 mg. For all subjects, calcium to phosphorus ratios were less than 1 and for four men and four women were about 0.5. In view of the serious health aspects of osteoporosis, especially in women, consumption of such low-calorie diets should be accompanied by calcium supplementation. Although the etiology of osteoporosis is not yet known, high phosphorus intakes have been reported to accentuate calcium depletion in animals (26). Therefore, a ratio closer to 1:1 would be desirable.

Iron-deficiency anemia has been extensively reported (27-29). In view of trends toward lower consumption of beef (4) and apparent low caloric intake of United States women (6), it is necessary for women to take supplementary iron to meet the RDA. In this study, no women met the RDA. The highest intake recorded was 89% of the RDA and the lowest was only 37%. Average intake was only 62% of the RDA.

A relatively unexpected find was that intakes of vitamin A in some women were below the RDA. Although one-half of the women consumed the RDA or better, three women consumed less than 50% and four consumed less than two-thirds the RDA. Since vitamin A intakes vary seasonally (30), the fact that diets were recorded in winter could present an inaccurate picture of yearly intake levels. In view of dangers associated with excess intakes of vitamin A (31), recommendations for this vitamin should be limited to an increased intake of foods high in vitamin A rather than vitamin supplementation.

Cholesterol intakes were low. According to the dietary records, they averaged 375 mg for men and women combined. Estimated intake from food disappearance data is 576 mg/day, although in a previous study we found an average to be 446 mg (32). The P/S ratio was slightly higher in our present study than either in estimated average ratios (8) or in the previous study (32). This could reflect the increasing belief of the United States'

population in the association between cholesterol and heart disease (33-35) and saturated fat intake and heart disease (34, 35).

Meal patterns reflected a wide range of differences. Average percent of intakes at three time periods and number of meals per day did not vary between men and women. The majority of calories was consumed in the evening (52%), indicating a trend toward increased caloric intake in the evening (32, 36). The number of meals consumed averaged 3.26 for women and 3.57 for men and ranged from 1 to 6/day. This is slightly less than data from a previous study which found subjects to consume 3.8 meals/day (32). This may indicate a trend toward gorging, eating large amounts of calories at one time or in few meals, which is considered to be more efficient in converting calories to body fat (10) and would not be desirable in a society concerned with overnutrition and obesity.

These results indicate that there may be a large discrepancy in amounts of food recorded as eaten in a dietary record and those consumed during a controlled period. The reason or reasons for these huge discrepancies are not known. The types and numbers of different foods are probably accurate. If caloric intakes recorded by the women are accurate and/or representative, they may have difficulty in obtaining necessary amounts of calcium, vitamin A, and iron without dietary changes or vitamin and mineral supplementation.

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Synopsis Article

PHYSICAL GROWTH OF EXCLUSIVELY BREAST FED-INFANTS

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SUMMARY AND CONCLUSIONS

Weight and length were measured in a group of 222 infants, 104 boys and 118 girls, exclusively breast-fed and followed prospectively from birth to 9 months of age. The average anthropometric measurements of breast-fed infants were comparable to the National Center for Health Statistics standards for the first three months but slightly lower subsequently, particularly beyond 6 months of age. Morbidity experience indicated a marked benefit of breast feeding, especially reflected in incidence of respiratory and ear infections and diarrhoeal illness. Reduction in illness was seen also in those whose weight-for-age was in the lower centiles of NCHS standards. It is suggested that NCHS standards are not appropriate for evaluating the growth performance of breast-fed infants and that growth curves based exclusively on breast-fed infants should be used for that purpose. Physical growth slightly less than the average NCHS standard may be optimal for infants who are exclusively or predominantly breast-fed.

KEY WORDS: Breast feeding, growth, infections, diarrhoea, infant feeding.

OBSERVATIONS

This synopsis article is an extension of studies previously reported (1,2). For details of subject selection, demographic data, definitions, and methods, reference should be made to earlier publications. The morbidity experience and growth of a large group of exclusively breast-fed infants followed prospectively are described in Tables 1 and 2.

TABLE 1

MORBIDITY EXPERIENCE

	Breast-fed (n=222)	Formula-fed (n=235)	Significance p
Respiratory infection	1.02*	2.59	< 0.01
Otitis media	0.24	2.36	< 0.001
Diarrhoea	0.08	0.43	< 0.01
Dehydration	0.02	0.17	< 0.01
Pneumonia	0.02	0.14	< 0.01
Urinary tract infection	0.10	0.16	NS

*Number of episodes per 100 child-weeks. NS - not significant

TABLE 2

Physical growth of breast-fed infants compared with NCHS data

AGE		BOYS percentiles 5th	50th	95th		GIRLS percentiles 5th	50th	95th
Birth	BF	2.55	3.23	4.09	Weight	2.40	3.24	3.87
	NCHS	2.54	3.27	4.15	(kg)	2.36	3.23	3.81
	BF	46.1	50.7	54.8	Length	45.5	50.0	52.7
	NCHS	46.4	50.5	54.4	(cm)	45.4	49.9	52.9
1 month	BF	3.24	4.36	5.42	Weight	2.93	4.04	4.90
	NCHS	3.16	4.29	5.38	(kg)	2.97	3.98	4.92
	BF	50.3	54.8	58.2	Length	49.5	53.8	56.5
	NCHS	50.4	54.6	58.6	(cm)	49.2	53.5	56.9
3 months	BF	4.50	5.79	7.14	Weight	4.31	5.25	6.68
	NCHS	4.43	5.98	7.37	(kg)	3.18	5.40	6.74
	BF	56.9	60.8	65.0	Length	55.6	59.3	63.0
	NCHS	56.7	61.1	65.4	(cm)	55.4	59.5	63.4
6 months	BF	5.91	7.41	9.10	Weight	5.44	6.80	8.14
	NCHS	6.20	7.85	9.46	(kg)	5.79	7.21	8.73
	BF	62.9	67.5	72.1	Length	61.4	65.7	70.1
	NCHS	63.4	67.8	72.3	(cm)	61.8	65.9	70.2
9 months	BF	6.99	8.51	10.15	Weight	6.58	7.98	9.39
	NCHS	7.52	9.18	10.93	(kg)	7.00	8.56	10.17
	BF	67.9	71.7	76.7	Length	65.9	70.3	74.6
	NCHS	68.0	72.3	77.1	(cm)	66.1	70.4	75.0

BF Breast-fed NCHS National Center for Health Statistics data

The number of breast fed infants examined was 104 boys and 118 girls from birth to 4 months, 100 boys and 112 girls at 5 and 6 months, and 92 boys and 104 girls from 7 to 9 months of age. The data are based on singleton full-term neonates of white caucasian mothers belonging to middle and upper socioeconomic strata.

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EFFECT OF PROTEIN MALNUTRITION AND TUMOR GROWTH ON SERUM TOTAL COMPLEMENT, C3, AND IgG LEVELS DURING E. COLI INFECTION IN THE RAT

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ABSTRACT

This study was undertaken to observe the changes in total hemolytic complement (THC), complement 3 (C3), and immunoglobulin G (IgG) levels during E. coli infection in control rats, control rats with tumor (CT), protein-depleted rats (PD), and protein depleted rats with tumors (PDT). Serum THC levels were lower in the infected, PD rats than in the uninfected, PD rats. The PD rats had lower C3 levels compared with the other groups. Serum IgG levels decreased in all groups, but decreased more in the PD rats than in the control rats. The presence of infection in the control, CT, PD, and PDT rats reduced IgG levels compared to the uninfected rats in these groups. The results suggest that protein depletion depressed the complement and IgG response more than the presence of the tumor.

KEY WORDS: Protein depletion, tumor growth, humoral immunity, total hemolytic complement, C3.

INTRODUCTION

The loss of immunocompetence with a subsequent lowered resistance to infections frequently occurs in malnourished children (1-3) and cancer patients (4-6). Protein malnutrition is also a complicating factor in the cancer patient. The specific contributions of tumor growth and malnutrition to the altered immunological response of the host are not known.

Studies on the effects of protein deprivation on the serum globulin and complement components of the immune system have produced conflicting

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results. Investigators have shown that immunoglobulins and/or complement levels were decreased (2,3) or unaltered (1) in malnourished children. In addition, serum IgG levels were also depressed in malnourished dogs while others (7) reported that IgG levels were unaltered in protein or food-restricted guinea pigs (8). McGhee et al. (9) found no change in serum complement levels in rat mothers fed a low protein diet. However, serum complement levels were reduced in their offspring compared to litters from normal rats. More recently, Peterson et al. (10) and Wunder et al. (8) showed that C3 levels decreased in protein-deprived rats and guinea pigs.

Tumor growth in cancer patients and tumor-bearing animals also affects the immune system of the host. With respect to the complement system, investigators (11,12) have found that serum complement levels were elevated in cancer patients and in tumor-bearing rats. Recently, Nishioka et al. (12) found that complement levels increased in cancer patients during the early stages of the disease but declined during the late stage.

From the reports on clinical observations, it has become apparent that these studies are limited by the heterogeneity of the patient population, the type of malignancy, the presence of infections, the degree of protein malnutrition, and other nutrient deficiencies. An animal model is required to study the interrelationship of tumor growth, protein malnutrition, and infection. Use of an animal model has the advantages of separating out the co-existing variables by quantitating protein depletion and tumor size, as well as defining the type of infection. This study, utilizing a rat tumor-model, was designed to observe the separate and combined effects of protein depletion and tumor growth on the levels of total complement, C3, and IgG.

METHODS

Male, Lewis (LEW/MAI) (Microbiological Associates, Walkersville, MD) rats weighing between 230-270 g were used in this study. Prior to the start of the experiment, the rats were fed Purina Rat Chow (Ralston Purina Co., St. Louis, MO) and water ad libitum. On day 8, all rats were assigned to four groups of 12 rats each: control rats, CT rats, PD rats, and PDT rats. Groups CT and PDT were injected subcutaneously in the dorsal area with 1.5×10^6 cells from a rat mammary adenocarcinoma (AC33). The rats in the non-tumor-bearing groups were injected in a similar manner with saline. The AC33 tumor was initially induced in Lewis rats during the testing of dimethyl-B-aziridinopropionamide as a possible anti-lymphoma drug (13). The above dose of AC33 adenocarcinoma grew rapidly following the induction stage; 100% of the rats died in 4-5 weeks after inoculation (Miller, E. E., unpublished observation). Food consumption was equalized in all groups by feeding a maintenance amount of 15 g per day. In a previous pilot study, we found that rats fed the protein-free diet consumed on the average of 15 g per day. This amount of powdered rat chow was offered to the rats in the control groups. The protein-depleted groups were fed a similar amount of a protein-free diet which was isocaloric to the chow diet (Table 1). Food intake was recorded daily. Animals were housed individually in metabolic cages at $23 \pm 1^{\circ}C$ on a 12 hour light-dark cycle. On day 13, half of the rats in each group were inoculated with E. coli. The E. coli-treated rats were injected between 10 and 11 a.m. via the lateral tail vein with

1.1 X 10^9 live colonies of ATCC 25922 E. coli strain. The concentration of viable organisms was determined by the pour plate technique in duplicate. Twenty-four hours later, all rats were killed by decapitation. Tumors were removed and weighed. Final carcass weights of tumor-bearing rats were calculated by subtracting the tumor weights from the total body weights. Blood obtained from cardiac puncture was cultured for the presence of E. coli organisms in tryptic soy broth and incubated overnight at 37°C. Trunk blood was allowed to clot at 4°C and the serum was separated by centrifugation and stored at -70°C. Livers were excised, weighed, and stored at -20°C for determination of protein (14), glycogen (15), and fat by the Folch method (16) as modified by Milner (17).

TABLE 1

Protein-Free Diet

Ingredient	Amount (%)
Corn Starch	46.0
Dextrin	22.0
Sucrose	15.0
Corn Oil	6.0
Fiber[1]	3.8
Minerals[2]	5.0
Vitamins[3]	2.2

[1]Solka floc, Brown Co., Berlin, New Hampshire.
[2]Rogers and Harper Salt Mixture #902842, ICN Pharmaceuticals, Inc., Cleveland, Ohio.
[3]Vitamin Fortification Mixture #904654, ICN Pharmaceuticals, Inc., Cleveland, Ohio.

Serum total hemolytic complement (THC) levels were assayed with antibody-coated sheep erythrocytes (18). The degree of hemolysis of diluted serum was determined spectrophotometrically. The amount of hemolytic complement activity was calculated and expressed as a percent of total hemolysis after subtracting the hemolysis in an unsensitized, cell control in order to rule out non-specific, cell damage. Concentrations of serum C3 were measured by the radial diffusion method described by Mancini (19) using goat anti-rat C3 and rat C3 for the standards (Cappel Laboratories, Cochranville, PA) in 2% agarose containing a glycine buffer with 0.04 M EDTA. Serum IgG levels were also determined by radial diffusion (19) using the rat IgG kit (Miles Laboratories, Inc. Elkhart, IL). The remainder of the serum was analyzed for total protein (21) and albumin (21). Total serum globulins were calculated as the difference between the values for total protein and albumin (22).

Analysis of variance and linear regression were performed as described by Ott (23). Statistical significance between means were determined by using the least significant difference procedure (23). Differences between means were considered significant at $P < 0.05$.

RESULTS

All E. coli-infected rats were alive 24 hours after injection and their blood cultures were positive for E. coli organisms. Food intake,

body weight change, and tumor weights are presented in Table 2. Food intake was not significantly different among the four groups. During the 14 day period body weights decreased in groups CT, PD, and PDT by 11%, 20%, and 18%, respectively. All tumors were palpable by day eight. The tumor weights of the CT rats fed the chow diet were 72% larger than the rats in group PDT fed the protein-depleted diet. These differences in tumor weights may be caused by the depletion of host tissues in the rats fed the protein-free diet thus limiting the supply of nutrients for the tumor. This observation has been reported by several investigators (24-27). The CT rats lost more carcass weight than the control rats. In contrast, carcass weights were similar in the PDT and PD rats. Although carcass composition was not measured in the present study, the rats in the PDT group may have had more carcass water which would mask the differences in carcass weight between these two groups.

TABLE 2

Food Intake, Body Weight, and Tumor Weight of Protein-Depleted and Control Rats With and Without Tumors

Groups	Food Intake	Initial Body Weight	Final Body Weight	Tumor Weight
	(g)	(g)	(g)	(g)
C	14.7 ± 0.2^{1a}	258 ± 3^{a}	259 ± 3^{a}	
CT	14.5 ± 0.4^{a}	253 ± 4^{a}	226 ± 5^{2b}	34.6 ± 2.3^{a}
PD	14.5 ± 0.5^{a}	252 ± 3^{a}	202 ± 3^{c}	
PDT	14.4 ± 0.4^{a}	256 ± 4^{a}	211 ± 4^{2c}	9.6 ± 0.4^{b}

[1]Values are means ± SEM for 6 rats. Means not sharing a common superscript in the same column are significantly different ($P<0.05$).
[2]Final body weights for groups CT and PDT are carcass weight-tumor weight.

The mean changes in serum THC, C3, and IgG concentrations are shown in Table 3. THC levels decreased by 13% in the infected rats fed the protein-free diet compared to the uninfected rats in the same dietary groups. The bacterial challenge did not affect the levels of THC in the control, CT, or PDT rats. Also, there were no significant differences in the THC levels among the uninfected rats in any of the groups. Consequently, THC levels were not affected by tumor growth in this study. In contrast, C3 levels decreased by 33% in the rats fed the protein-free diet compared to the control rats. There were no significant differences in the C3 levels during infection in groups CT, PD, or PDT. These data suggest that protein-depletion for 14 days has a greater effect on C3 levels than the presence of the tumor or the E. coli infection. C3 levels were reduced more during protein depletion (33%) than during infection (15%) compared to the levels of the control group. On the other hand, IgG levels of all experimental groups were less than the mean level of the control group. IgG levels decreased in the infected control, CT, PD, and PDT rats when compared to the uninfected rats in these groups by 52%, 73%, 34%, and 49%, respectively.

TABLE 3

Serum Complement and IgG Levels During E. Coli Infection in Protein-Depleted and Control Rats With and Without Tumors

Groups		Total Hemolytic Complement	Complement 3	IgG
		% hemolysis	mg/ml	mg/100ml
C	Uninfected	88.2±0.7[1a]	0.66±0.03[a]	446.0±49.7[a]
	Infected	97.6±1.8[a]	0.56±0.02[b]	214.5±20.2[b]
CT	Uninfected	96.7±2.6[a]	0.76±0[a]	322.6±32.7[c]
	Infected	89.1±0.4[ab]	0.69±0.05[a]	85.6± 8.5[d]
PD	Uninfected	97.1±4.4[a]	0.44±0.03[c]	219.2±21.2[b]
	Infected	84.2±3.0[b]	0.48±0.04[bc]	145.4± 7.9[bd]
PDT	Uninfected	91.4±6.0[ab]	0.45±0.03[c]	193.8±25[b]
	Infected	85.9±4.0[b]	0.52±0.04[bc]	99.3±5.3[d]

[1]Values are means ± SEM for 6 rats. Means not sharing a common superscript(s) in the same column are significantly different ($P < 0.05$).

Relative to the control rats, serum total proteins and albumin levels were reduced in all groups (Table 4). Tumor growth produced a 24% decrease in total protein in groups CT and PDT compared to the control group; whereas protein depletion lowered total protein by 12% compared to the control rats. Albumin levels were lowest in the PDT group while globulins decreased by more than 30% in the tumor-bearing rats when compared to the controls.

Liver weights decreased significantly in the PD and PDT groups compared with the control group (Table 5). The liver protein content of all experimental groups was significantly less than the control group. The E. coli infection produced a decrease in the protein content of the control and CT groups and an increase in liver protein of the PD group compared to the uninfected rats in these groups. Liver glycogen was significantly less in the infected than in the uninfected rats in groups PD and PDT. Glycogen content was also reduced in the infected, control rats but the differences were not significant compared to the control group. In contrast, the protein-depleted rats in groups PD and PDT exhibited an excessive accumulation of liver glycogen. Liver fat content was not significantly altered during infection in groups CT, PD, and PDT. However, the infected, control rats had 24% more liver fat than the control rats.

TABLE 4

Serum Proteins During E. Coli Infection in Protein-Depleted and Control Rats With and Without Tumors

Groups		Total Protein	Albumin	Globulins
		g/dl	g/dl	g/dl
C	Uninfected	5.9 ± 0.1^{1a}	3.4 ± 0.1^{a}	2.5 ± 0.1^{a}
	Infected	5.0 ± 0.2^{b}	2.8 ± 0.0^{b}	2.1 ± 0.3^{a}
CT	Uninfected	4.6 ± 0.1^{c}	2.7 ± 0.1^{b}	1.9 ± 0.1^{b}
	Infected	4.5 ± 0.1^{c}	2.9 ± 0.1^{b}	1.5 ± 0.1^{c}
PD	Uninfected	5.2 ± 0.1^{b}	2.8 ± 0.0^{b}	2.3 ± 0.1^{a}
	Infected	5.2 ± 0.1^{b}	2.9 ± 0.1^{b}	2.3 ± 0.2^{ab}
PDT	Uninfected	4.5 ± 0.2^{c}	2.8 ± 0.1^{b}	1.7 ± 0.1^{c}
	Infected	4.5 ± 0.1^{c}	2.2 ± 0.2^{c}	2.0 ± 0.1^{abc}

[1]Values are means ± SEM for 6 rats. Means not sharing a common superscript(s) in the same column are significantly different ($P < 0.05$).

TABLE 5

Liver Composition During E. Coli Infection in Protein-Depleted and Control Rats With and Without Tumors

Groups		Weight	Protein	Glycogen	Fat
		(g)	mg/g	mg/g	mg/g
C	Uninfected	9.4 ± 0.2^{1a}	145 ± 3^{a}	18.7 ± 0.6^{ab}	31.2 ± 1.6^{ac}
	Infected	9.4 ± 0.5^{a}	97 ± 4^{b}	11.7 ± 0.6^{a}	38.7 ± 2.4^{b}
CT	Uninfected	10.2 ± 0.4^{ac}	113 ± 4^{c}	7.3 ± 0.8^{a}	28.8 ± 0.9^{cd}
	Infected	10.6 ± 0.6^{c}	78 ± 1^{d}	27.3 ± 1.6^{b}	26.2 ± 0.8^{d}
PD	Uninfected	7.2 ± 0.1^{bd}	80 ± 3^{d}	86.8 ± 7.9^{c}	34.6 ± 0.8^{ab}
	Infected	6.3 ± 0.1^{b}	125 ± 3^{c}	18.0 ± 1.1^{ab}	34.2 ± 1.8^{ab}
PDT	Uninfected	7.5 ± 0.2^{d}	124 ± 5^{c}	62.3 ± 7.9^{d}	33.1 ± 1.0^{ac}
	Infected	7.1 ± 0.1^{bd}	121 ± 3^{c}	17.7 ± 3.6^{ab}	29.7 ± 1.4^{cd}

[1]Data are means ± SEM, N=6; means not sharing a common superscript letter in the same column are significantly different ($P < 0.05$).

DISCUSSION

To our knowledge, this is the first study to suggest that malnutrition may depress the immune system of the malnourished, tumor-bearing host more than the presence of the tumor. Our results have shown that THC levels were lower in the infected than uninfected, protein-depleted rats. The reduced levels of THC may be caused by an impairment in the secretion of the complement proteins from the liver as the liver protein content was higher in the infected rats than in the uninfected rats on the protein-free diet. Similar results were reported on malnourished children (2,28) with infections. On the other hand, THC levels were not altered in the uninfected, protein-depleted rats. These findings were in agreement with those of Kenny et al. (29) and Sakamoto et al. (30) who used rats that were malnourished for two to four weeks. The presence of this tumor in the chow fed or protein-depleted rats did not alter THC levels at 14 days after inoculation of the tumor. Yet, complement levels may not be affected at this stage of tumor growth. Weimen et al. (31) pointed out that complement levels in rats began to rise from the levels of the control rats after 19 days of tumor growth and continued to increase until death.

Serum C3 levels were significantly reduced in the PD and PDT rats compared to the control rats which suggests that synthesis of C3 is impaired. Contrary to the results of McCabe (32), who reported no change in C3 levels between controls and patients with gram-negative bacteremia, our study showed that C3 levels of the E. coli-treated, control rats were lower than the uninfected, control rats. The presence of E. coli infection did not further decrease C3 levels in the CT, PD, and PDT rats compared to the uninfected rats in these groups. It may be possible that substances with complement activity are released during this stage of protein depletion and/or tumor growth which would maintain or increase C3 levels in response to the additional stress of infection (31). Furthermore, it is also possible that C3 is generated through the action of lysosomal enzymes released as a consequence of protein malnutrition.

Serum IgG levels were depressed more in the uninfected, PD and PDT rats compared to the uninfected, CT or control rats. Dionigi et al (7) reported in dogs while Aref et al. (33) and Chandra (2) reported in children that IgG levels were reduced during malnutrition. The results of this present study are in agreement with their findings. Other studies involving malnourished children (34) and adults (1) have found an increase in IgG levels compared to control subjects. The elevation of IgG levels in these studies may reflect the presence of infection in these subjects (2). In the present study, IgG levels of all groups were not elevated 24 hours after infection. It is possible that IgG levels may increase at a later time point in the course of the infection.

The results of this study suggest that protein malnutrition leads to an impaired complement and IgG response in this tumor-bearing, rat model when challenged with E. coli infection. The presence of protein malnutrition in the host may stress the functioning of the immune system more than the growing tumor. These observations further support the merits of nutritional therapy in improving the immune response of the cancer patient.

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THE CHOLINE REQUIREMENT OF THE GROWING KITTEN IN THE PRESENCE OF JUST ADEQUATE DIETARY METHIONINE[1]

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ABSTRACT

This study was conducted to determine the level at which choline is required by the growing kitten fed a 33% soy protein diet containing methionine just at its required level and total sulfur amino acids in excess. All experimental diets were isocaloric with a calculated metabolizable energy of 5.0 kcal/g. The soy protein used in each diet contributed 0.04% choline by analysis, and six levels of added choline were studied: 0, 0.10, 0.15, 0.20, 0.25, and 0.30g choline per 100g of diet. Kittens were fed the diets for 30 days in a 4 x 6 randomized block design, and food intake and body weights were recorded three times weekly. At the end of the feeding period, the liver was removed and analyzed for water content and total lipids, and a heparinized blood sample was taken for subsequent analysis of plasma methionine, albumin, total protein, glutamate-oxaloacetate transaminase (GOT), glutamate-pyruvate transaminase (GPT) and γ-glutamyl transpeptidase (GGT) activities. Weight gain and liver lipid response curves exhibited a plateau at approximately 0.2% added choline. GOT and GPT activities were significantly higher in the plasma of kittens fed the diet with no added choline than in any other of the groups. However, activities of both enzymes in that group were within normal range. No effect of dietary choline concentration was seen on any other plasma parameter measured. Assuming that the bioavailability of the choline contributed by the dietary protein is 100%, data presented indicate that 0.24% choline in a diet of 33% protein and 5.0 kcal/g, is adequate to support maximal weight gain and minimal liver fat in the growing kitten, when methionine is present just at its required level, and total sulfur amino acids are present in excess.

KEY WORDS: Choline, animal nutrition, methionine.

INTRODUCTION

Considerable species variability exists in the extent to which choline is required in the diet of mammals. The magnitude of the requirement for choline is inversely related to the amount of choline (or phosphatidyl choline) that can be

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synthesized by the species in question, and that depends, at least in part, on the rate of methylation of ethanolamine (or phosphatidylethanolamine). Although little research into the choline requirement of the feline has been published, evidence in the literature indicates that choline is required in the diet of the growing kitten for maximal growth and minimal liver fat when methionine is included in the diet just at the level of its requirement (1), and possibly when methionine is present in excess of its requirement (2). Published work does not, however, permit an accurate estimation of a recommended dietary level of choline. The best estimate in the literature of a requirement for maximal growth and minimal liver fat in the kitten (1) describes a range of greater than 0.1% but probably less than 0.3% of the diet.

The purpose of the experiment reported here was to more precisely estimate the choline requirement of the kitten when methionine is provided in the diet just at its required level, but total sulfur amino acids (methionine plus cystine) are present in the diet in excess of the requirement (3,4,5). Mammals respond to choline deficiency with development of fatty liver, which appears to precede growth depression in the time course of the response to a choline inadequate diet. Total liver lipids and weight gain were therefore measured as the principle response criteria for estimating the choline requirement of the kitten. Some mammals respond to choline deficiency with alterations in plasma protein metabolism, but the nature and degree of the response is quite variable, and appears to depend on species, as well as to some extent on age and sex of the animal, and on duration of the deficiency state (6,7,8,9,10). Mansur Guerios and Hoxter (10) reported that with prolonged choline deficiency, young male cats developed severe hypoalbuminemia. We therefore measured plasma albumin and total protein to evaluate their usefulness in estimating the kitten's choline requirement. Plasma glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) and γ- glutamyl transpeptidase (GGT), were also measured since presence of those enzymes in the plasma at elevated concentrations is indicative of liver damage in some species, and disruption of cellular integrity may occur with development of fatty liver. Finally, plasma free methionine was determined to evaluate the impact, if any, of indequate choline intake on maintenance of normal plasma free methionine levels.

MATERIALS AND METHODS

Animals

Twenty four specific pathogen-free domestic shorthair kittens, 18 males and six females, with an average initial weight of 1200 g, were used in this experiment. Kittens were vaccinated against panleukopenia and were housed individually in stainless steel cages, with food and water available ad libitum throughout the experiment. From weaning until the beginning of the experiment, kittens were fed the basal soy protein diet shown in Table 1.

Experimental Diets

Six levels of choline were fed in soy protein diets similar to the basal diet, except that choline bitartrate (Sigma Chemical Co., St. Louis, MO) was used instead of choline chloride, and the soy protein was supplemented with cystine instead of methionine. Methionine was replaced by cystine in order to keep the choline sparing capacity of the diet via dietary methionine to a minimum. Methionine was provided by the soy protein at a level of 0.4% of the diet, and cysteine at a level of 0.43% of the diet (analysis by Ralston Purina). Total sulfur amino acids (methionine plus cystine) then, including the 0.6% added cystine, constituted 1.43% of the diet. Microbiological assay of the isolated soy protein indicated a total choline content of 1145 μg/g, as analysed by Raltech Scientific Services, Madison, WI, according to the method of Horowitz and Beadle (11). The soy protein, then, at 33% of the diet, contributed 0.04% choline to each experimental diet. The fat contained less than

38 µg choline per g, and contributed less than 0.0009% choline. Choline bitartrate was included in the experimental diets at 0.0, 0.21, 0.31, 0.42, 0.52 and 0.63%, to provide an additional 0.0, 0.1, 0.15, 0.2, 0.25 and 0.3% of the diet as free choline. Although starch content was varied slightly with the level of choline, the six experimental diets were essentially isocaloric at a calculated metabolizable energy of approximately 5.0 kcal/g.

TABLE 1
Composition of Basal Diet

Component	% of Diet
Soy protein[1]	33.0
Chicken fat[2]	25.0
Cerelose[3]	15.0
Salt mix[4]	4.0
Vitamin mix[5]	1.0
Methionine[6]	0.6
Starch[7]	21.1
Choline chloride[8]	0.3
Solka floc	1g/100g complete diet

[1]RP-100, Ralston Purina Co., St Louis, Mo.
[2]Stewing chicken fat, Valley Fresh, Turlock, CA; heat rendered + 0.02% BHT
[3]Cerelose, dextrose, 2001, Corn Products, Englewood Cliffs, N.J.
[4]Supplied, per kg diet: g/100g mineral mix: $CaHPO_4$, 47.4; K_2HPO_4, 10.0; $CaCO_3$, 13.0; $MgSO_4$, 5.04; KCl, 7.5; NaCl, 13.9; $MnSO_4 \cdot H_2O$, 0.48; $ZnSO_4 \cdot 7H_2O$, 0.556; $CuSO_4 \cdot 5H_2O$, 0.10; $FeC_6H_5O_7 \cdot 3H_2O$, 0.60; $Ca_5(IO_6)_2$, 0.018; $SnCl_2 \cdot 2H_2O$, 0.01; Na_2SeO_3, 0.003; $(NH_4)_6Mo_7O_4 \cdot 4H_2O$, 0.005; $CrCl_3 \cdot 6H_2O$, 0.032; $NiCl_2 \cdot 6H_2O$, 0.03; NaF, 0.014; $NH_4VO_3 \cdot 4H_2O$, 0.0025.
[5]Supplied, per kg diet: retinyl palmitate, 20,000 IU; cholecalciferol, 2000 IU; DL- α-tocopheryl acetate, 160 IU; menaquinone, 15 mg; thiamin-HCl, 25 mg; riboflavin, 10 mg; pyridoxine-HCl, 10 mg; nicotinic acid, 100 mg; calcium pantothenate, 20 mg; myo-inositol, 200 mg; folic acid, 10 mg; cyanocobalamin, 50 µg; biotin, 1 mg; ascorbic acid as a preservative for thiamin, 200 mg; taurine, 100 mg.
[6]Vega Biochemicals, Tucson, Arizona
[7]Melojel, food grade cornstarch, National Starch and Chemical Co., Bridgewater, N.J.
[8]ICN Pharmaceuticals, Inc. Cleveland, Ohio

Design

A 4 x 6 randomized block design was used. All kittens in each block were of the same sex and began receiving the experimental diets simultaneously. Four blocks of six kittens became available over a 3 1/2 month period. The initial mean body weights (+ standard error) of each of the four blocks of six kittens were 1610 + 98, 1070 + 99, 1098 + 179, and 1022 + 164. Kittens were fed the experimental diets for 30 days, during which period food intake and body weights were recorded three times weekly. At the end of this period, a blood sample was taken into heparinized syringes from the jugular vein and immediately placed on ice. The kittens were then euthanized with sodium pentobarbital and immediately upon death, the liver was removed, freed of connective tissue, placed on ice, and frozen for later analysis. Blood samples were centrifuged, and plasma was separated and frozen for later analysis.

Livers were freeze-dried and extracted with petroleum ether (30-60 b.p.) for 7 days in a soxhlet apparatus. Total petroleum ether soluble material was taken as liver lipid, and lipid content was measured as weight loss after ether extraction. Percentage total liver lipid on a dry and wet weight basis was then calculated. Plasma free methionine concentration was determined as previously described (12).

Plasma albumin was measured colorimetrically with bromocresol green binding (Sigma, Kit no. 630, St. Louis, Mo., 63178). Plasma total protein was determined by refractive index (Refractometer, A/O, TS Meter, American Optical Corp., Keen, New Hampshire). Activities of plasma GOT, GPT and of GGT were assayed according to the methods of Karmen (13), Wroblewski and LaDue (14) and Szasz (15) respectively. Data was subjected to an analysis of variance, and when indicated, differences between means were evaluated with Tukey's Omega Procedure (16).

RESULTS

The effects of dietary choline concentration on weight gain, food intake and total liver lipid of kittens are shown in Table 2 and Fig. 1. Kittens fed the diet with no added choline did not gain weight during the 30 day period. There was a linear dose-response to choline of weight gain up to the level of 0.2% added choline (r=0.99); more than 0.2% added choline had no beneficial effect on weight gain. Weight gain of kittens fed the diet with no added choline was significantly less than weight gain of kittens fed any of the diets with added choline ($p < 0.05$).

No significant differences occurred among mean food intakes of the six groups of kittens ($p > 0.05$); however, the lowest food intake occurred in kittens fed the diet with no added choline.

Minimal liver lipid content was attained in kittens fed the diet containing 0.2% added choline. There was an inverse linear dose-response (r=0.99) of wet liver lipid content to dietary choline concentration from 0.0% to 0.2% added choline. Total lipid contents of wet livers of kittens fed 0.0, 0.10, and 0.15% added choline were not significantly different; however, these values were significantly greater than those of kittens fed the 0.20, 0.25 and 0.30% added choline diets. The pattern of response of total lipid in dry liver to dietary choline concentration was similar to the wet tissue data; however, significant differences were not present due to greater variability in this parameter.

No effect of dietary choline concentration was seen on plasma albumin, methionine, total protein, or GGT (see Table 3). GOT and GPT concentrations were significantly higher ($p < 0.01$) in kittens fed the diet with no added choline than in groups fed every other level of choline.

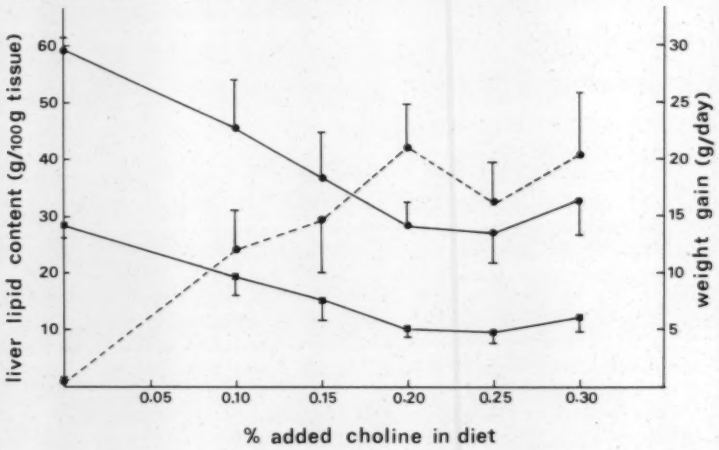

FIG. 1

Effect of dietary choline concentration on liver lipids, wet weight (■—■), liver lipids, dry weight (●—●) and on weight gain (●--●) in kittens. (means ± standard errors)

TABLE 2

Effect of Dietary Choline Concentration on Food Intake, Weight Gain, and Total Liver Lipids[1]

	% choline in diet					
	0.0	0.10	0.15	0.20	0.25	0.30
Food intake (g/day)	34 ± 3[a]	44 ± 8[a]	46 ± 12[a]	65 ± 6[a]	51 ± 12[a]	58 ± 12[a]
Weight gain (g/day)	0 ± 0[2]	12 ± 4[a]	15 ± 5[a]	21 ± 4[a]	16 ± 4[a]	20 ± 6[a]
Total liver lipid						
g/100g wet tissue	28.5 ± 2.2[a]	19.5 ± 3.4[a]	15.2 ± 3.9[a]	10.0 ± 1.4[b]	9.8 ± 2.5[b]	12.0 ± 2.5[b]
g/100g dry tissue	59.5 ± 1.7[a]	45.6 ± 8.5[a,b]	36.9 ± 7.8[a,b]	28.2 ± 4.06[b]	27.0 ± 5.5[b]	32.8 ± 6.2[a,b]

[1] Values (means + standard errors) within response criterion not sharing common superscript are significantly different, $p < 0.05$.
[2] Calculated value was - 0.08 ± 0.29. All weight gain values are rounded off to the nearest gram.

TABLE 3

Effect of Dietary Choline Concentration on Several Plasma Parameters[1]

	% choline in diet					
	0.0	0.10	0.15	0.20	0.25	0.30
Albumin (g/dl)	3.18 ± 0.35	3.63 ± 0.24	3.4 ± 0.1	3.5 ± 0.1	3.2 ± 0.1	3.4 ± 0.1
Total protein (g/dl)	5.5 ± 0.8	6.2 ± 0.3	5.6 ± 0.2	5.8 ± 0.1	5.5 ± 0.1	5.6 ± 0.2
Methionine (nmol/ml)	29.0 ± 3.8	31.4 ± 9.9	22.8 ± 3.8	21.0 ± 1.8	29.7 ± 3.4	22.7 ± 3.4
GOT (IU/L)	39.5 ± 6.6[a]	13.7 ± 1.5	9.5 ± 0.8	10.0 ± 1.6	13.0 ± 1.5	9.8 ± 2.1
GPT (IU/L)	12.7 ± 0.9[a]	4.0 ± 1.1	2.8 ± 1.0	3.0 ± 0.9	4.0 ± 0.8	6.0 ± 0.7
GGT (IU/L)	6.0 ± 2.2	4.0 ± 0.9	7.0 ± 4.5	2.0 ± 1.7	3.5 ± 1.8	5.0 ± 3.5

[1] Values given are means ± standard errors [a] Significantly different ($p < 0.01$) from other means within response criterion.

DISCUSSION

The National Research Council currently recommends that choline be included in the diet of the feline at the level of 0.2 g per 100 g of a diet providing 4.0 kcal/g (15). Reports of experimental work done with the feline that would provide a solid basis for this recommendation, however, are lacking in the literature. In 1958, Carvalho da Silva et al. (2) produced choline deficiency in cats fed a 42% casein, 2.5% gelatin diet containing no added choline or vitamin B_{12}. Cats fed this diet with 0.1% added choline grew only slightly better than and had the same level of liver fat as the cats fed the choline-free purified diet. With 0.5% choline, improvements in weight gains and liver fat levels were seen, but neither parameter approached the level of response seen with control cats fed a "natural" diet. Cats fared no better with respect to liver fat with 10% raw beef liver added to the purified choline-free diet than cats fed the choline-free diet, but they grew as well as the cats fed 0.5% choline. It was concluded that with the purified diet used, 0.1% choline was inadequate for maximal growth and minimal liver fat. However, since maximal growth and minimal liver fat were not attained when 0.5% choline was added to the diet, it may be concluded that either 0.5% choline was inadequate, or that the diet was inadequate with respect to other nutrients, or possibly that both conditions existed. Methionine and vitamin B_{12} should be considered as important nutrients, relative to choline nutriture, that might be inadequate in their diet. Although methionine is indeed the limiting amino acid in casein, the principle protein source used in the purified diet, at 42% of the diet, casein would provide approximately 1.07% methionine and 0.13% cystine[2], a level of total sulfur amino acids well in excess of the kitten's requirement (3,4,5). As the authors indicated (2) their purified diet contained no added vitamin B_{12}. However, supplementing the purified diet with 10% beef liver, which according to the authors, supplied approximately 0.5 ug of B_{12} per day, provided no advantage over the 0.5% choline diet with respect to weight gain, (i.e., cats still grew submaximally, though better than those fed the choline-free or 0.1% choline purified diets). Cats fed the diet containing raw beef liver had liver lipid levels equivalent to those of the cats fed a choline-free diet. These results could be interpreted to mean that while the liver supplement did provide a growth stimulating factor absent in the purified diet, the cats were still choline deficient (at 10% of the diet, the added liver would provide no more than 0.047% choline) (17), despite apparently adequate dietary vitamin B_{12} and methionine. Moreover, it is possible that even cats fed the purified diet with no added B_{12} source were not B_{12} deficient. Cats were prefed for 3 weeks a diet providing ample B_{12} from raw beef heart, and it is unlikely that 8 weeks on a deficient diet would entirely deplete B_{12} stores. Also, forty two percent crude casein would probably provide approximately 1/3 the dietary level of B_{12}[3] recommended by the NRC (18). While one cannot draw a conclusion from this experiment with respect to the level of choline required by the kitten, the experiment as reported does provide evidence that choline is a dietary essential for the cat, and may provide some insight into the question of choline-methionine interactions in the feline, as will be discussed later in this paper.

A report published by Mansur Guerios and Hoxter (10) described severe hypoalbuminemia associated with prolonged choline deficiency in kittens. The hypoalbuminemia improved upon addition of 0.5% choline to the diet, but was not prevented by inclusion of an additional 0.5% D,L-methionine in the choline-free diet. Once again, while this report does not provide information as to the level of choline required in the diet of the kitten, it does provide evidence that choline is a required nutrient, and again, could throw some light on the matter of choline-methionine interaction.

Recently, Anderson et al. (1) conducted experiments both to determine the

[2]As calculated from "Amino acid content of food and biological data on proteins," FAO, 1970. (Rome).

[3]As calculated from vitamin B_{12} concentration of High Nitrogen Casein (U.S. Biochemicals), which is reported to contain 1.3 ug/g protein.

choline requirement of the kitten when methionine was provided just at its required level in a crystalline amino acid diet, as well as to investigate the capacity of dietary methionine to spare choline in the feline. Kittens were fed diets containing either no choline, 0.05% or 0.10% choline for 14 days. A fourth diet, choline free but made isomethyl to the 0.1% choline diet with 0.37% additional methionine, was also fed. Of the three levels of choline tested, 0.1% choline resulted in the best weight gain in kittens, and 0.37% additional methionine in place of 0.1% choline promoted the same rate of gain. Liver lipid concentrations were not determined in this experiment. In a separate experiment, weight gain and liver lipid concentrations were compared in two groups of cats fed either 0.1% or 0.3% choline. While the two groups gained weight at the same rate, the mean liver lipid concentration of the group fed 0.3% choline was lower than that of the group fed 0.1% choline. It may be concluded from these data that while 0.1% choline may support maximal growth in kittens, it is not adequate for minimal liver fat. One cannot conclude however, that 0.3% choline is adequate, since no plateau in response was demonstrated.

We have tested the effect of six levels of free choline, 0.0, 0.1, 0.15, 0.2, 0.25, and 0.3%, added to diets containing 0.04% choline by analysis (contributed by isolated soy protein), on weight gain and liver lipid concentrations in the kitten. As can be seen in Fig. 1, plateaus in the response curves of both parameters occur at approximately 0.2% added choline. There is considerable disagreement in the literature as to the bioavilability of choline present in the protein in natural diet ingredients such as soy bean protein (19-23). In estimating the requirement, however, it will be assumed that the choline present in the protein is 100% available in order to avoid underestimation of the requirement. Since the protein contributes 0.04% choline to the experimental diets, our results indicate that 0.24% of the diet as choline is adequate to maintain minimal liver fat as well as maximal rates of gain in rapidly growing kittens.

Although a comprehensive species comparison of choline requirements is not within the scope of this paper, it is of interest to note that the choline requirement of the rapidly growing rat fed marginal methionine is similar to that of the kitten. Young et al. (24) fed rapidly growing rats a diet mildly deficient in methionine and total sulfur amino acids, and found that 0.16% choline was adequate to maintain both minimal liver fat and maximal growth when fed in a diet of 15% protein and 15% fat. Hale and Schaefer (25) attempted to determine the choline requirement of young rats with a diet just adequate in methionine and cystine and containing 21% protein and 20% fat. The highest level of choline fed was 0.2% of the diet, and that level resulted in the lowest concentration of liver fat. Their results indicate that the requirement is at least 0.2% of the diet, and may be greater, since no plateau in liver lipid response to dietary choline concentration was demonstrated. Considering the numerous dietary and physiological variables which can affect the choline requirement of an animal, e.g., levels of dietary fat and protein, fatty acid chain length and degree of saturation, dietary caloric content, growth rate and age (25-29), the similarity between requirements of the growing rat and the kitten is worthy of note.

We have examined a number of plasma parameters in order to evaluate the impact of dietary choline concentration on plasma protein metabolism, plasma methionine homeostasis, and liver cellular integrity. Some species appear to respond to choline deficiency with aberrations in protein metabolism, but the nature of the response is quite variable. Fischer and Garrity (8) reported that serum albumin decreased to below normal after one week of choline deficiency in 22-23 day old female rats, and α-globulin levels increased significantly. The effect on serum albumin was not observed in older male rats in later work by the same group (9). Agostini and Alfisi (6) found total serum protein to be decreased on the seventh day of feeding a choline-free diet to rats, but observed an increase in albumin levels. Synthesis of serum protein does not appear to be abnormal in dogs fed a choline-free diet for six weeks, when compared to dogs fed the same diet with added choline (7). Mansur Guerios and Hoxter (10) reported that cats submitted to prolonged choline deficiency, i.e., 9 months of feeding a 34% casein diet, or 3 months of feeding a 17% casein diet, exhibited a severe depression in serum albumin concentration. In the present experiment, however, after 30 days of choline deficiency, albumin and total protein concentrations in plasma of kittens were normal. It is possible that the

duration of deficiency in our experiment was insufficient for the hypoalbuminemia response to be observed, or that the purified choline-free diet of Mansur Guerios and Hoxter may not have been fully adequate in other respects.

Despite varied levels of dietary choline, and the presence of choline deficiency, plasma free methionine levels were maintained at a normal level by each group of kittens in this experiment. If methyl group transfer from methionine was stimulated by a choline deficiency, it might be expected that plasma methionine levels would decrease. It appears clear then, that when methionine is included in the diet just at its required level, plasma methionine homeostasis and the need for this amino acid for protein synthesis are priority functions in the kitten over that for choline synthesis.

Disruption of liver cell integrity to such an extent that a significant increase was seen in plasma levels of GOT and GPT, occurred only in the most severely choline deficient state, at a point when close to 30% of the liver wet weight was lipid. However, even the highest plasma levels were still within the normal range for the cat (30). Plasma concentrations of GGT were unaffected by the degree of choline deficiency created in this experiment. Determination of these plasma enzyme concentrations, therefore, is of little if any value in the assessment of a choline deficient state in the kitten.

The work reported herein confirms that choline is a dietary essential for the kitten when the diet fed provides methionine just at its required level and an excess of total sulfur amino acids. The question of whether or not concentrations of dietary methionine exceeding the requirement for this amino acid for protein synthesis can minimize or eliminate the dietary requirement for choline in the kitten is not fully resolved. As described earlier in this paper, Carvalho da Silva et al. (2) produced choline deficiency in kittens by feeding them a choline-free diet that provided sulfur amino acids in amounts that exceed the requirement of the kitten (3,4,5). Their purified diet contained no added vitamin B_{12}, and a deficiency of that vitamin could prevent endogenous synthesis of choline. However, as described earlier, despite the fact that B_{12} was not added to the diet, it is unlikely that the kittens were B_{12} deficient. Moreover, when a supplement of 10% raw beef liver, rich in Vitamin B_{12}, was added to the choline-free diet, liver lipids did not decrease from the maximum level that was seen with a choline-free diet. These data, therefore, could be interpreted to indicate that the transfer of methionine methyl groups for the formation of choline does not occur in the kitten to an extent great enough to meet the requirement for choline.

A subsequent report by Mansur Guerios and Hoxter (10) provides some interesting though incomplete information on the question of methionine and choline interaction. In examining the effect of choline deficiency on serum protein synthesis, the authors fed kittens either a 34% or 17% casein choline-free diet; no information beyond this was given about the diets fed. Hypoalbuminemia was seen after 3 months of feeding the 17% casein diet, but hypoalbuminemia appeared only after 9 months of feeding the 34% casein diet. Hypoalbuminemia was responsive to choline supplementation on both diets, but responded only after 53 days of choline supplementation of the 17% casein diet. Methionine would have been inadequate in the 17% casein diet, and present in just adequate levels in the 34% casein diet. This difference in methionine concentrations may account for the difference in response to the diets. However, in a separate experiment, the addition of 0.5% D,L-methionine to the 34% casein diet did not prevent the decline in serum albumin concentrations, whereas substitution of 0.5% choline for the methionine resulted in elevation of serum albumin and a substantial increase in weight gain. This latter result might indicate that methionine cannot provide methyl groups sufficient for choline biosynthesis in the kitten, even when methionine is present at concentrations adequate for protein synthetic needs. However, since no information is provided on the Vitamin B_{12} status of the kittens, firm conclusions concerning choline-methionine interaction cannot be drawn.

Anderson et al. (1) on the other hand, conclude on the basis of their data that dietary methionine in excess of the level required by the kitten for protein synthesis is capable of completely sparing choline. Kittens fed a crystalline amino acid diet just adequate in methionine and containing 0.1% choline exhibited a rate of gain

identical to that of kittens fed a choline-free diet containing an additional amount of L-methionine isomethyl to 0.1% choline. When fed in the presence of 2-amino-2-methyl-1-propanol, a compound shown to inhibit choline biosynthesis in the rat when fed in a diet containing no choline (31), the additional methionine did not afford any advantage to the kittens in terms of their weight gains, when compared to the kittens fed the choline-free diet containing the inhibitor. Their results provide evidence that the synthesis of choline utilizing methyl groups from methionine does take place in the kitten, at least to the extent that rates of gain equivalent to what is seen with 0.1% choline can be achieved with additional methionine alone. Our results indicate, however, that choline is required in the diet of the kitten at the level of 0.24% when methionine is included in just adequate concentrations. Further investigation is required to determine if the entire choline requirement for both maximal weight gain and minimal liver fat can be met by the provision of excess dietary methionine.

In this context, it is interesting to note that in the work of Young et al. with young rats (24), a wide range of efficiency of utilization of methionine for lipotropic activity was demonstrated. At very low concentrations in a choline-free diet, methionine appeared to be used quite effectively as a lipotrope, but its lipotropic potency decreased as its concentration in the diet increased. Moreover, even the highest concentration of methionine fed did not result in normal liver fat levels. This phenomenon, as well as other aspects of dietary composition, should be carefully considered in any investigation of the capacity of methionine to spare choline in the diet.

CONCLUSION

Total liver lipid and growth rate were the most useful of parameters measured in this experiment for the assessment of the choline requirement of the kitten in a short term study of choline deficiency. On the basis of these two parameters, the minimum dietary choline requirement of the kitten appears to be 0.24% of a diet containing approximately 5.0 kcal metabolizable energy per gram. This estimate was made with a diet containing methionine just sufficient to meet the requirement for this amino acid for protein synthesis, but containing excess total sulfur amino acids (methionine and cystine).

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UPTAKE AND ABSORPTION OF ZINC IN PERFUSED RAT JEJUNUM: THE ROLE OF ENDOGENOUS FACTORS IN THE LUMEN

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ABSTRACT

These studies tested whether jejunal uptake and absorption of zinc in the rat occurs in the absence of soluble endogenous factors (including putative Zn-binding ligands) in the lumen. Using isolated segments of the adult rat proximal jejunum in situ, washed free of luminal contents and perfused with a recirculating glucose-saline medium containing 20 μM Zn+^{65}Zn, we observed rapid mucosal uptake and efficient transmural transport of ^{65}Zn. Similar experiments using a non-recirculating single-pass perfusion actually revealed a somewhat greater uptake of ^{65}Zn making it unlikely that a factor essential for ^{65}Zn uptake was being secreted into the lumen by the tissue itself. The uptake and absorption of ^{65}Zn, ^{67}Cu, and ^{109}Cd in this system were all consistent with in vivo studies. We conclude that endogenous luminal factors are not an essential component of the Zn uptake system. The enhancement of Zn absorption in the intact animal by some low molecular weight Zn-binding ligands may therefore be due to their interaction with dietary Zn resulting in a more absorbable form of the metal, rather than due to replacement of a missing component of the uptake system.

Key Words: Zinc, Intestinal Absorption, Jejunum, Zinc-binding Ligands

INTRODUCTION

Much evidence has accumulated to suggest that the extent of intestinal absorption of zinc is greatly influenced by the nature of the metal-binding species which are simultaneously present in the gut (1). Dietary phosphate, fiber, phytate and several other metal-binding substances, for example, form coordination complexes with Zn which markedly restrict absorption of this metal (2-6). However, other ligands, such as amino acids, citrate, and hydroxyquinolines appear to enhance Zn absorption (3, 7-10). Moreover, Evans and colleagues (11-13) have postulated that a specific Zn-binding ligand, possibly of pancreatic origin (14) and thought to be picolinic acid, is secreted into the upper intestinal tract and is required for normal absorption of zinc.

The essentiality of such an endogenous ligand could derive from either or both of two possible functions: (1) The ligand could

serve as an endogenous "liberating agent" for dietary Zn, favorably competing with non-absorbed dietary Zn-binding ligands, thereby making the metal available for absorption, and (2) The ligand could be required as an indispensable component of the transport system for Zn, perhaps acting somewhat in the manner of a co-factor. It might be imagined, for example, that a co-factor could orient the substrate (Zn) in the active site of the appropriate membrane carrier.

We tested the second possibility by studying the mucosal uptake and transmural transport of Zn by the isolated rat jejunum, maintained in situ, washed free of residual contents, and perfused through the lumen with a saline medium containing no added metal-binding ligands. The jejunum was selected for these studies because, even though Zn absorption occurs throughout the length of the small intestine (1), the length of the jejunum relative to that of the duodenum and its position proximal to the ileum suggest that a major portion of dietary Zn is absorbed by the jejunum. The uptake and absorption of ^{67}Cu and ^{109}Cd were also measured in this system to obtain a more complete picture of metal transport under these conditions.

METHODS

Adult male Sprague Dawley rats (Charles River Breeding Laboratories, Portage, MI) weighing 175 to 250 g were maintained on Purina Laboatory Chow (Ralston Purina Co., St. Louis, MO) and tap water ad libitum. The rats were fasted with free access to drinking water 24 h prior to surgery. Under Inactin anesthesia (110 mg/kg body weight, i.p.; BYK Gulden Konstanz, West Germany), a 6-15 cm segment of the proximal jejunum, starting at the Ligament of Treitz, was isolated without compromising the mesenteric circulation, as previously described (15). Body temperature was maintained at 37° by a heating pad regulated by a rectal probe. The isolated segment was first perfused with approximately 10 ml of 5 mM glucose in 0.15 M NaCl without recirculation, to remove luminal contents. The segment was then perfused at 0.8 ml/min with the glucose-saline medium containing 20 μM $ZnSO_4$, $CdCl_2$, or $CuCl_2$, and 0.1-1.0 μCi of tracer ^{65}Zn, ^{109}Cd (New England Nuclear, Boston, MA), or ^{67}Cu (Atomic Energy of Canada, Ltd., Ottawa), respectively.

In studies where the perfusate was recirculated through the lumen, the total perfusate volume was 1.6-2.0 ml and 0.1-1.0 μCi polyethylene glycol (PEG, $M_r \sim 4000$, New England Nuclear) labelled either with ^{14}C (for counting with ^{65}Zn) or ^{3}H (for ^{67}Cu and ^{109}Cd) was included as an impermeant volume marker. Aliquots of 10-50 μl of the perfusate were periodically taken for analysis by liquid scintillation spectrometry. Rates of metal removal were calculated from changes in the metal:PEG ratio as previously described (15).

In single-pass perfusion studies, the perfusate was introduced into the lumen at 0.8 ml/min and the effluent was collected in separate fractions at 1.0-min intervals. The transit time of perfusate through the segment was measured by timing the passage of a bolus of blue dextran (Pharmacia Fine Chemicals, Piscataway, NJ).

After perfusion for 25 min, the lumen was washed with glucose-saline and the tissue segment was removed. Remaining perfusate, washings, and the tissue were counted in a well-type gamma counter. The rate of transfer of metal into the body was then calculated from the amount of isotope removed from the perfusate minus that recovered in the tissue. For ^{65}Zn and ^{67}Cu, the rates of absorption determined in this way represent minimum values since the dilution of the

isotope by exchangeable Zn and Cu within the tissue is neglected (see Ref. 16). Because Cd is transferred from intestinal tissue into the body at a very low rate (17), absorption of Cd was also measured in tissue perfused for 25 min and washed as above, but maintained in situ for 275 min before being removed for analysis. The mean tissue wet weight per unit length under these conditions did not differ from that of tissue analyzed after only 25 min of perfusion.

RESULTS AND DISCUSSION

^{65}Zn was rapidly and progressively removed from the recirculating jejunal perfusate over a 30-min period (Fig. 1). The average rate of net lumen-to-mucosa transfer of Zn was comparable to that for Cd (15, 17) and Cu (Table I). The further transport of Zn into the body was rapid, occurring at a rate of at least 48% of that of mucosal uptake. Similar results were obtained for Cu, whereas Cd, a toxic element generally regarded as non-essential, was largely retained within the intestinal tissue (Table I and Ref. 17).

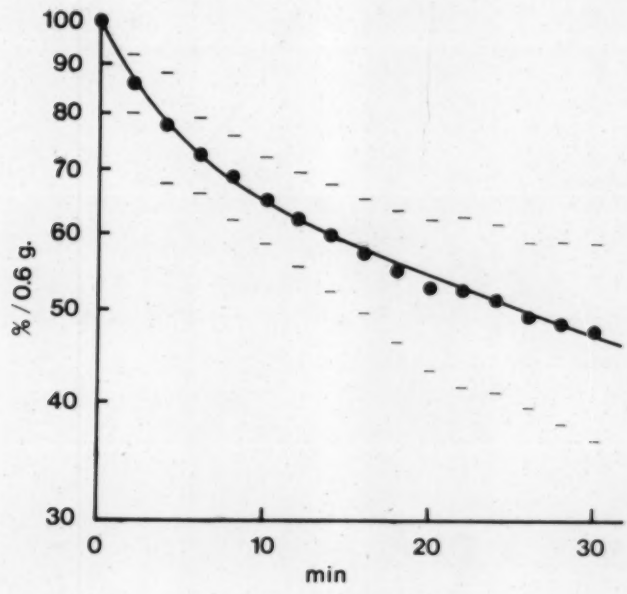

FIG. 1

Time course of ^{65}Zn removal from a recirculating luminal perfusion of the proximal jejunum of adult rats (N=5). The perfusate initially contained 20 µM Zn and 5 mM glucose in 0.15 M NaCl. Flow rate was 0.8 ml/min. The data are expressed as the mean ± SD percent of initial ^{65}Zn remaining in the perfusate, normalized to 0.6 g wet weight of perfused tissue, the mean tissue weight used in these studies.

TABLE I

Rates of Metal Transport in Perfused Rat Jejunum*

	Zn	Cu	Cd
Removal from perfusate (I)	1.05±.20	1.50±.40	1.88±.80
Transfer to body (II)	0.50±.13	0.71±.24	<0.02
II/I (%)	48	47	<2

*nmol/gm-min

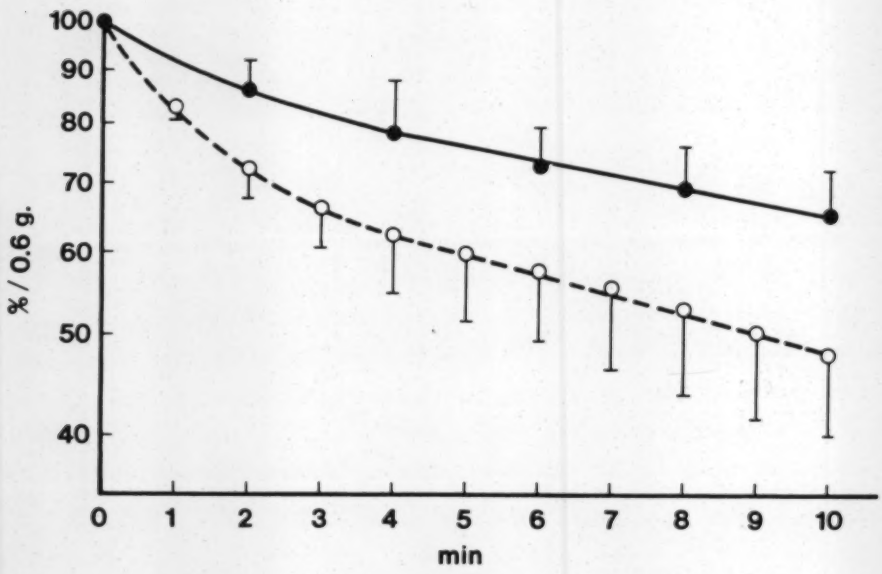

FIG. 2

Comparison of ^{65}Zn removal from (●) recirculating and (o) single-pass perfusions. Rates of removal in single-pass perfusions were determined from the $^{65}Zn/^{14}C$ ratio in fractions of the effluent collected at 1.0-min intervals. These rates were then applied to a hypothetical recirculating perfusion to facilitate a direct comparison with those observed in an actual recirculating perfusion (Fig. 1). Points indicate the mean ± SD of the percent of initial ^{65}Zn remaining in the perfusate per 0.6 g tissue, determined in 5 recirculating and 3 single-pass preparations. The curves are significantly different from each other ($p<.015$) as determined by fitting a series of orthogonal polynomials within a repeated measures design.

The observed rapid uptake of all three metals, the efficient absorption of Zn and Cu (6, 18-20), and the retention of Cd within the intestinal tissue (6, 17, 21-22) are all consistent with earlier findings by other investigators, thus indicating that the isolated tissue preparation used in the present studies behaves "normally" with respect to these metals. But as the perfused jejunal segment was isolated from secretions of other regions of the digestive tract and carefully washed to remove its contents, and as the tissue was perfused with a ligand-free medium, the above findings indicate that the mucosal uptake and transmural transport of Zn and Cu do not require the presence in the lumen of factors derived from other regions of the digestive system. Similar results for Zn absorption were obtained by Antonson et al. (23) who also used saline media for in situ perfusion.

The possibility remains, however, that an essential factor is secreted into the lumen of the perfused segment by the isolated tissue itself. We tested this for Zn by comparing the above findings with results of similar studies wherein the perfusate made only a single pass through the isolated tissue segment. Under these circumstances, putative factors essential for Zn uptake could not accumulate in the perfusate and thus a diminished uptake for ^{65}Zn would likely be observed. The results (Fig. 2), wherein the measured removal rates have been transformed to allow a direct comparison with the data in Fig. 1, reveal that there is actually a significantly greater ($p<.015$) removal of ^{65}Zn from the non-recirculating perfusion. These results therefore fail to support the hypothesis that a metal-binding ligand (or other factor) is secreted into the jejunal lumen which functions as an essential component of the transport system for Zn. We conclude that the "essentiality" of such putative factors could therefore only stem from a requirement to enhance the bioavailability of dietary Zn from foodstuffs.

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EFFECTS OF DIETARY LIMITATIONS OF HISTIDINE, METHIONINE, OR THREONINE ON THE SUSCEPTIBILITY OF MICE TO INFECTION WITH SALMONELLA TYPHIMURIUM AND LISTERIA MONOCYTOGENES

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ABSTRACT

The effects of dietary limitations of histidine, methionine, or threonine on the susceptibility of mice to Salmonella typhimurium and Listeria monocytogenes were investigated. Weanling CF1 or SW mice fed diets limited in 75% histidine or threonine but not methionine were more susceptible to S. typhimurium. Mice fed diets limited in 75% methionine or threonine were more susceptible to L. monocytogenes. Mice from two of the experimental dietary groups challenged with 6 times the 50% lethal dose of S. typhimurium cells on day 5 after immunization with 10^8 heat killed cells exhibited a lower resistance than the control group, however, by day 8 after immunization, all dietary groups developed adequate resistance. The increased susceptibility of mice to S. typhimurium was reversed by replenishing the diet after 2 weeks with the limiting amino acid, histidine or threonine. The replenishment of the diet was not effective to reverse the increased susceptibility of mice to L. monocytogenes. The increased susceptibility to L. monocytogenes is likely to be due to the impairment of mouse cell mediated immunity which is not reversible by dietary replenishment.

KEY WORDS: amino acid malnutrition, susceptibility to infection, mouse cell-mediated immunity, replenishment of the diet

INTRODUCTION

Human subjects, especially children, as well as experimental animals on protein or amino acids malnourished diets are more susceptible to infections, disease, and death (1-3). Supplementation of protein deficient diets with a mixture of essential amino acids increase the resistance to infection in experimental animals (3,4). Nutritional deficiency may be a result of improper diet or the effect of the infection itself (5,6). Factors which contribute to the host resistance against microbial infections include humoral immunity (specific antibody production; 7,8), cell mediated immunity (CMI; 9), and nonspecific factors such as phagocytosis (10). Thus the increased susceptibility of protein or

amino acids malnourished hosts may be due to the impairment of one or more of these host defense mechanisms.

Experimental diets used to investigate the effects of dietary limitations of essential amino acids upon the susceptibility to infection have consisted of protein sources such as gluten, soya α-protein, or less than 10% casein which are known to be deficient in one or more of the essential amino acids, as well as diets prepared by mixing amino acids in proportions found in casein (11,12). To prepare experimental diets, it is important to maintain total nitrogen balance and optimum concentrations of essential amino acids for a specific young, developing host species (13).

Petro and Bhattacharjee (14) developed an experimental system in which weanling mice were fed semisynthetic diets having total nitrogen balance and essential amino acids at optimum for murine development. In this system it was possible to demonstrate the effects of 50% dietary limitations of leucine, isoleucine, valine, or lysine on the levels of serum proteins including immunoglobulins, and the susceptibility of mice to Salmonella typhimurium and Listeria monocytogenes as well as on the levels of specific antibody and CMI (14,15). We report here the effects of dietary limitations of nonbranched chain amino acids, methionine, histidine, or threonine on the susceptibility of mice to S. typhimurium and L. monocytogenes as well as the effects of replenishment of limiting essential amino acid on the host susceptibility.

MATERIALS AND METHODS

Mice, diet and feeding regimen

Female, weanling (each 10-12 gm in weight), CF1 mice from Charles River Breeding Laboratory, Wilmington, Mass., and Swiss Webster (SW) mice from Cox Farm, Indianapolis, Ind., were used. The control diet consisted of all murine essential amino acids adjusted up to optimal dietary concentrations needed to achieve maximum growth (14,16). Each experimental diet limited in methionine, histidine, or threonine possessed same nitrogen and caloric content as the control diet and 25% (75% limited) or 50% (50% limited) of the limiting amino acid present in the control diet. Extra supplement of nonessential amino acids were added to each experimental diet to compensate for the loss of nitrogen from limiting the single essential amino acid. Mice were fed ad libitum specified diets for three weeks before injection with S. typhimurium or L. monocytogenes. Amount of food consumed and the body weight gain per day of individual mice were determined.

Bacterial cultures and experimental infection of mice

Maintenance of stock cultures of S. typhimurium SL3770 (courtesy of Dr. R. W. Treick) and L. monocytogenes (courtesy of Dr. A. A. Blazkovec), preparation of cell suspension, and the determination of exact number of cells injected were carried out according to the procedures published by Petro and Bhattacharjee (15). After three weeks on a specified diet, at least ten CF1 or SW mice from each dietary group were injected intraperitoneally (IP) with an appropriate number of S. typhimurium or L. monocyto-

genes cells. Mortality of mice was monitored for 14 days.

Immunization and challenge of mice with S. typhimurium

After three weeks on a diet, mice from specific dietary groups were immunized IP with heat killed 10^8 cells of S. typhimurium SL3770. On day five and eight after immunization, at least ten mice per dietary group were challenged IP with approximately 6 times the 50% lethal dose of viable S. typhimurium SL3770 and the mortality was recorded for 21 days.

Replenishing the diet with the limiting amino acid

Mice from specific dietary groups were put on experimental diets for two or three weeks after which the limiting essential amino acid was replenished and mice were fed the control diet. All groups of mice were injected after three weeks with S. typhimurium or L. monocytogenes, and the mortality rate was observed for 14 days.

Statistical analysis

Significance of the difference in mortality was determined by chi-square analysis and values at $p \leq 0.05$ were taken as significant.

RESULTS

Susceptibility of mice fed essential amino acid limited diets to experimental infection with either S. typhimurium or L. monocytogenes

Mice fed 50% methionine, histidine, or threonine limited diets for three weeks exhibited lower weight gain than mice fed the control diet. Mice fed 75% methionine, histidine, or threonine limited diet exhibited little or no weight gain indicating the establishment of nutritional limitations in each of the experimental dietary groups of mice.

The CF1 mice fed diets limited in 50% methionine, histidine, or threonine for three weeks exhibited no significantly different mortalities following injection with S. typhimurium SL3770 than mice fed the control diet. However, CF1 mice fed 50% valine (15), or 75% histidine limited diet exhibited a significantly higher mortality after injection with S. typhimurium SL3770 than CF1 mice fed the control diet (Table 1). Similarly, SW mice fed diets limited in 75% histidine or threonine exhibited significantly higher mortality than SW mice fed the control diet. By contrast, CF1 and SW mice fed diet limited in 75% methionine did not exhibit significantly higher mortality than CF1 and SW mice fed the control diet. There were 10^8 colony forming units (CFU) of S. typhimurium in the liver and spleen of each dead mouse (15).

SW mice were used in subsequent experiments. Mice fed diet limited in 75% methionine or threonine exhibited a significantly higher mortality in two experiments after injection with L. monocytogenes than SW mice fed the control diet (Table 2). The SW mice fed histidine limited diet exhibited a significantly higher mortality in experiment I but not in experiment II.

TABLE 1

Effect of Dietary Essential Amino Acid Limitations on the Susceptibility of Mice to S. typhimurium SL3770[a]

Dietary limitation	Strain of Mouse: CF1 No. injected	CF1 % Mortality	SW No. injected	SW % Mortality
None	12	42	16	25
75% Methionine	16	50	15	47
50% Valine	12	83[b]	ND[c]	--
75% Histidine	14	93[b]	16	63[b]
75% Threonine	ND[c]	--	15	73[b]

[a]After 21 days on specific diets, CF1 and SW mice were injected IP with approximately 50% lethal dose of S. typhimurium SL3770. Mortality was monitored for 14 days.

[b]Significantly different than mice fed the control diet.

[c]Not determined.

TABLE 2

Effect of Dietary Essential Amino Acid Limitations on the Susceptibility of Mice to L. monocytogenes[a]

Dietary limitation	Exp. I: SW mice injected with 4.9×10^5 cells No. injected	Exp. I % Mortality	Exp. II: SW mice injected with 1×10^6 cells No. injected	Exp. II % Mortality
None	15	20	10	60
75% Methionine	15	93[b]	10	100[b]
75% Histidine	18	67[b]	8	38
75% Threonine	15	93[b]	11	100[b]

[a]After 21 days on specific diets, SW mice were injected IP with the indicated number of L. monocytogenes cells. Mortality was monitored for 14 days.

[b]Significantly different than mice fed the control diet.

Effect of dietary limitation of essential amino acid on humoral immune response of mice against S. typhimurium

Immunization with heat killed S. typhimurium is known to elicit only a humoral immune response in mice (7,17). All dietary groups of immunized mice exhibited a significantly higher resistance to challenge with live S. typhimurium SL3770 cells on day five and eight after immunization than the nonimmunized control

mice (Table 3).

TABLE 3

Effect of Dietary Limitations of Histidine, Methionine, or Threonine on the Resistance of Mice to a Challenge with Viable S. typhimurium SL3770 Cells on Days 5 and 8 After Immunization[a]

Dietary limitation	Day 5 after immunization		Day 8 after immunization	
	No. challenged	% Mortality	No. challenged	% Mortality
None	17	0	20	15
75% Methionine	15	13	12	25
75% Threonine	13	38[b]	12	42
75% Histidine	11	17[b]	13	15
Control[c]	4	100[b]	4	100[b]

[a]After 21 days on the dietary regimen, SW mice were immunized IP with 10^8 heat killed S. typhimurium SL3770; mice were challenged 5 or 8 days later with approximately 6 times the 50% lethal dose of viable SL3770 cells. Mortality was monitored for 21 days.

[b]Significantly different than immunized mice fed the control diet.

[c]Mice were fed the control diet and injected without immunization with 6 times the 50% lethal dose of viable SL3770 cells; all deaths occurred by day 4 after the injection.

The SW mice fed histidine or threonine limited diet had significantly lower resistance to the challenge on day five after immunization than the immunized mice fed the control diet. Mice fed the control or methionine limited diets exhibited similar resistance. Experimental dietary groups of mice challenged on day eight after immunization did not exhibit significantly different levels of resistance than the immunized mice fed the control diet.

Effects of replenishing the diet with limiting amino acid on the susceptibility of mice to S. typhimurium and L. monocytogenes

Mice fed histidine or threonine limited diet exhibited significantly higher mortality to S. typhimurium injection than mice fed the control diet. However, mice fed the histidine or threonine replenished diet after two weeks did not exhibit significantly higher mortality than mice fed the control diet (Table 4). But mice fed histidine or threonine replenished diet after three weeks exhibited significantly higher mortality than mice fed the control diet. Mice fed methionine limited diet exhibited higher mortality to L. monocytogenes injection than mice fed the control diet (Table 5). Mice fed methionine replenished diet after two and three weeks also exhibited a significantly higher susceptibility to L. monocytogenes than mice fed the control diet. Replenishment of diet with threonine after two and three weeks also did not alter the susceptibility of mice to L. monocytogenes.

TABLE 4

Effect of Replenishing the Histidine and Threonine Limited Diets After Two and Three Weeks on the Susceptibility of Mice to Infection With S. typhimurium SL3770

Dietary limitation	Period on dietary limitation	Replenish after	No. injected with 1.4×10^5 cells	% Mortality
None	None	---	16	25
75% Histidine	All through the exp.	None	16	63[b]
75% Histidine	2 weeks	2 weeks	15	33
75% Histidine	3 weeks	3 weeks	15	60[b]
None	None	---	12	8
75% Threonine	All through the exp.	None	11	73[b]
75% Threonine	2 weeks	2 weeks	10	30
75% Threonine	3 weeks	3 weeks	10	70[b]

[a]The first group was fed control diet. The second group was fed 75% threonine or histidine limited diet. The third group was fed threonine or histidine limited diet for 2 weeks, after which the dietary limitations were replenished. The fourth group was fed limited diet for 3 weeks, after which the dietary limitation was replenished. After 21 days on the dietary regimen, all groups were injected IP with viable S. typhimurium SL3770 cells. Mortality was monitored for 14 days.

[b]Significantly different than mice fed the control diet.

TABLE 5

Effect of Replenishing the Methionine and Threonine Limited Diets After Two and Three Weeks on the Susceptibility of Mice to Infection With L. monocytogenes

Dietary limitation	Period on dietary limitation	Replenish after	No. injected	% Mortality
None	None	---	15	20
75% Methionine	All through the exp.	None	15	93[b]
75% Methionine	2 weeks	2 weeks	14	86[b]
75% Methionine	3 weeks	3 weeks	15	80[b]
None	None	---	12	83
75% Threonine	All through the exp.	None	12	58
75% Threonine	2 weeks	2 weeks	10	100
75% Threonine	3 weeks	3 weeks	10	100

[a]The first group was fed control diet. The second group was fed 75% methionine or threonine limited diet. The third group was fed 75% methionine or threonine limited diet for 2 weeks, after which the dietary limitations were replenished. The fourth group was fed the limited diet for 3 weeks, after which the dietary limitations were replenished. After 21 days on the dietary regimen, mice on methionine and threonine replenishment were injected IP with 4.9×10^5 and 7.1×10^5 viable L. monocytogenes cells, respectively. Mortality was monitored for 14 days.

[b]Significantly different than mice fed the control diet.

DISCUSSION

Resistance to L. monocytogenes is a CMI function, however, the resistance to S. typhimurium requires, although not exclusively, a humoral immune function (17,18). Defined diet and young mice offer a sensitive experimental system to establish specific dietary amino acid limitations and investigate their effects on susceptibility to infection of the host and its immune response (15).

Dietary limitations of 50% branched chain amino acids, leucine, isoleucine, or valine affects the susceptibility of mice to S. typhimurium and the development of early humoral immune response but neither the in vitro phagocytic activity nor the CMI (15). The lack of increased susceptibility of mice fed 50% histidine, methionine, or threonine limited diet to S. typhimurium suggests that this level of dietary limitations of nonbranched chain amino acids is sufficient to protect the host against certain infections. Malnutrition is generally classified as mild, moderate, and severe (19). The increased susceptibility of mice fed diets limited in 75% histidine or threonine to S. typhimurium and 75% methionine, histidine, or threonine to L. monocytogenes suggests that there exists a lower level of nonbranched chain amino acid requirements to impair the immune response and increase the susceptibility of mice. The observed increased susceptibility of specific experimental dietary groups of mice is due to the proliferation of CFU of S. typhimurium and L. monocytogenes in the liver and spleen and not due to any secondary effects of dietary limitations.

Dietary limitations of essential amino acid appears to be specific in that methionine limitation unlike histidine or threonine had no effect on the susceptibility. Early development of humoral immune response as determined by the resistance to challenge on day five after immunization is impaired in histidine and threonine dietary groups. Studies on the effects of protein or amino acids malnutrition on immune mechanisms have produced somewhat conflicting results in human subjects and experimental animals (12,15,20). Since resistance to L. monocytogenes is a CMI function (9,18), the increased susceptibility of mice fed amino acid limited diets to L. monocytogenes indicates an impairment of the CMI function. The increased susceptibility to L. monocytogenes may be related to the impairment of T-cell number or function (15, 21). The results of replenishment experiments suggest that the

impairment of resistance to S. typhimurium but not to L. monocytogenes can be treated by replenishing the diet with specific limiting amino acids. It is possible that the replenishment after two weeks is sufficient for the B-cell function to recover from impairment and to reverse the susceptibility of mice to S. typhimurium but not sufficient to recover the impairment of T-cell function and to reverse the susceptibility to L. monocytogenes.

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BIOAVAILABILITY TO RATS OF IRON AND ZINC IN CALCIUM-IRON-PHYTATE AND CALCIUM-ZINC-PHYTATE COMPLEXES

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ABSTRACT

The bioavailability of iron in Fephytate and Ca_3Fephytate and of zinc in Zn_2phytate and Ca_4Zn_2phytate was assayed in rats. Hemoglobin response was the same to the iron of Fephytate as the reference iron compound, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ but the response to iron of Ca_3Fephytate was significantly less than to both Fephytate and the reference. The zinc of Zn_2phytate and Ca_4Zn_2phytate promoted growth as effectively as did $ZnSO_4$. The femur bone zinc response to Ca_3Zn_2phytate and Zn_2phytate did not differ significantly but the femur zinc responses to both complexes were significantly less than to $ZnSO_4$. In contrast to iron, calcium in a preformed calcium-zinc-phytate complex appears to have no significant effect on the bioavailability to rats of the zinc in the complex.

Key Words: Ca_4Zn_2Phytate, Fephytate, Ca_3Fephytate.

INTRODUCTION

There is now strong experimental evidence that phytate, depending upon the phytate:zinc molar ratio, decreases the bioavailability of zinc (1,2). It has also been reported that high calcium in the diet enhances this action of phytate (1,2). An observation by Oberleas (2) suggested that this calcium effect may be a result of the formation of insoluble CaZnPhytate complexes. He was able to show a synergistic effect of calcium and zinc ions, which when present simultaneously in solution with phytate, act together to increase the quantity of metallic phytate precipitate.

In view of the above finding, coupled with the thought by some investigators that phytate in grains exists as a mixed metallic salt, the present work was undertaken to: (a) compare the bioavailability of the iron of Fephytate with that of Ca_3Fephytate to rats and (b) compare the bioavailability of the zinc of Zn_2phytate with that of Ca_4Zn_2phytate to rats.

MATERIALS AND METHODS

Preparation of monoferric phytate (Fephytate) and Ca_3Fephytate.

Monoferric phytate was prepared by a detailed procedure (3). To prepare the Ca_3Fephytate, 3 g Fephytate was dissolved in 250

ml distilled water and to this solution was added dropwise and with constant stirring a $CaCl_2 \cdot H_2O$ solution (4.15 g $CaCl_2 \cdot H_2O$ in 50 ml distilled water). The resultant precipitate was collected by centrifugation, washed with demineralized water, then acetone. The washed product was air dried for 3 hours and placed under vacuum over night at room temperature. The calcium:iron:phytate molar ratio of the analyzed product was 3.0:1.1:1.0.

Preparation of Zn_2phytate and Ca_4Zn_2phytate. The Zn_2phytate was prepared by the following procedure. First, 17 g of sodium phytate was dissolved in an acetate buffer solution (75 ml of 0.1M NH_4acetate + 300 ml of 0.04 M acetic acid). To this solution was added dropwise with constant stirring a solution of 1.66 g $ZnSO_4 \cdot 7H_2O$ in 75 ml of 0.04 M acetic acid. Ethanol was added slowly to 50% by volume. The resultant precipitate was collected by centrifugation, washed with ethanol, then acetone and air dried. Analysis of the product indicated a zinc:phytate molar ratio 2.2:1. To prepare Ca_4Zn_2phytate, 3 g of Zn_2phytate was suspended in 1200 ml of demineralized water and a solution of 5 g $CaCl_2 \cdot 2H_2O$ in 50 ml of H_2O was added dropwise. The precipitate was collected by centrifugation, washed with water, then acetone and dried in a vacuum. Analysis of the dried product indicated a calcium:zinc:phytate molar ratio 3.6:2.2:1.0.

Animal trials. Semipurified basal diets were described by Morris and Ellis (1). The iron basal diet contained less than 5 ppm iron and the zinc basal diet contained less than 1 ppm zinc. Iron or zinc phytate salts were added to the basal diet at the expense of glucose. Male, weanling Sprague-Dawley rats(CD strain^R, Charles River Colony, Wilmington, MA) weighing 40-50 g were used with 10 animals per diet treatment. The animals were caged in suspended stainless steel cages. Food and distilled water were supplied ad libitum. Hemoglobin response was the criterion of iron bioavailability. Ferrous ammonium sulfate was used as the reference. Growth and femur zinc response were criteria of zinc bioavailability. The reference zinc compound was $ZnSO_4 \cdot 7H_2O$. Iron and zinc bioavailability trials were of 3 and 4 weeks duration, respectively. Animals were weighed weekly.

Analytical procedures. Zinc, iron and calcium were determined by flame atomic absorption spectrophotometry (4). Phytate was determined by the method of Ellis et al. (5).

RESULTS

Iron bioavailability. The iron sources tested were the two synthetic products, Fephytate and Ca_3Fephytate, described in Methods. Enough of each test and reference iron source was added to the basal diet to supply 18 ppm of dietary iron. Hemoglobin values for the bioassay of iron bioavailability are shown in Table 1.

TABLE 1

Comparison of FePhytate and Ca_3FePhytate as Dietary Iron Sources for Rats

Iron Source[1]	Hemoglobin[2]
	g/dl
Basal	4.4 ± 0.8[a4]
$Fe(NH_4)_2(SO_4)_2$	10.5 ± 0.6[c]
Fephytate	10.6 ± 0.9[c]
Ca_3Fephytate[3]	7.9 ± 0.8[b]

[1]Basal <5 mg/kg diet, all others 18 mg/kg added Fe.

[2]Fed diets 3 weeks, ten rats per group.

[3]Molar ratios, phytic acid 1.0, Ca 3.0, Fe 1.1.

[4]Mean ± S.D., values that share common superscripts are not statistically different, $P < 0.05$.

TABLE 2

Comparison of Zn_2Phytate and Ca_4Zn_2Phytate[4] As Dietary Zinc Sources For Rats

Zinc Source	4-Wk Gain	Femur Zinc	
		Concentration[2]	Total
	(g)	(µg/g)	(µg/femur)
Basal[1]	20 ± 8[a3]	53 ± 4[a]	7.7 ± 0.4[a]
$ZnSO_4 \cdot 7H_2O$	199 ± 20[b]	196 ± 9[b]	64 ± 4[b]
Zn_2phytate	200 ± 16[b]	171 ± 10[c]	60 ± 3[c]
Ca_4Zn_2phytate	194 ± 7[b]	166 ± 11[c]	56 ± 4[c]

[1]No added zinc, <1 µg/g. All other diets contained 12 µg/g of zinc as the respective compound. There were ten rats per group.

[2]Dry basis.

[3]Mean ± S.D., values that share common superscripts are not statistically different by Duncan's multiple range test, $P \leq 0.05$.

[4]Molar ratios by analysis Ca 3.6, Zn 2.2, phytic acid 1.0.

Hemoglobin values of rats fed Fephytate as an iron source equaled hemoglobin values of rats fed $Fe(NH_4)_2(SO_4)_2$ as an iron source. But the hemoglobin response of rats fed Ca_3Fephytate as an iron source was significantly less than that of both reference and Fephytate.

Zinc bioavailability. Growth and bone zinc response to Zn_2phytate and a mixed Ca_4Zn_2phytate salt is shown in Table 2. Both complexes supported a growth rate equal to the reference zinc compound. The Zn_2phytate and the mixed Ca_4Zn_2phytate were equivalent in support of femur zinc content, but the response to both were significantly less than the response to the reference zinc compound.

DISCUSSION

The poor bioavailability of the iron of Ca_3Fephytate, in comparison to Fephytate, is probably due to the low solubility. Monoferric phytate is readily soluble in aqueous solution whereas, Ca_3Fephytate is insoluble. Although Ca_4Zn_2phytate is also insoluble in aqueous solution, zinc of this complex supported growth as effectively as that of Zn_2phytate and $ZnSO_4$. These results suggest that there was sufficient dissociation of the zinc in both zinc-phytate complexes for adequate growth. In contrast to iron-phytate complexes, unpublished data in our laboratory indicate that zinc-phytate complexes dissociate at pH's below 3.9. Therefore, dissociation of the zinc phytate complexes in vivo would be expected since the pH of the gastric juice is below 3.9. The low response of the femur to the zinc of the phytate complexes in comparison to $ZnSO_4$ might suggest only partial dissociation of the zinc in the phytate complex.

In summary, calcium in a preformed Ca_3Fephytate complex decreased the bioavailability of the iron in the complex. In contrast, calcium in a preformed Ca_4Zn_2phytate complex appears to have no significant effect on the zinc utilization by rats.

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COMPARISON OF POLYETHYLENE GLYCOL AND DYE MARKERS IN NUTRITION RESEARCH

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ABSTRACT

Polyethylene and dye markers were compared as instruments for nutrition research. Such substances may function as representative markers for the liquid and particulate phase of gastrointestinal contents. Liquid phase markers have the greater diveristy of use. Particulate phase markers are limited in use to estimates of digesta movement and feces marking. When comparing marker types, polyethylene materials, as liquid or particulate phase markers, appear to be the marker of choice in the majority of studies. While neither marker, polyethylene or dye, are completely adequate for such research, the greater erractic recovery of dye markers reduces their effectiveness. Regardless of the marker used, it seems imperative that the researcher understand both the capabilities and limitations of the marker chosen. Furthermore, each investigator should validate his own indicator under his conditions for study.

KEY WORDS: Polyethylene Glycol, phenol red, dye markers, liquid phase markers, particulate phase markers.

The diversified use of markers, and of marker types, has resulted in the accumulation of considerable amounts of information. Many factors, including marker application, kinetics, reliability, etc., are discussed in various papers associated with this workshop. In the present paper we are interested in comparing polyethylene glycol and dye markers as instruments for nutritional research. We must, however, compare these markers on two levels of use; (I) as representative markers for the liquid phase of gastrointestinal contents, and (II) as markers representing the particulate phase of the ingesta. The two areas will be discussed as separate entities although polyethylene glycol and various dye markers can be, and frequently are, used as markers for both phases of gastrointestinal contents, liquid and particulate.

LIQUID PHASE MARKERS. Numerous markers have been used which fit into the category of "dye markers". Kindel (1) published a report on some 23 such fecal dye markers, and several others not mentioned in his report can be found in the literature (2, 3). However, in the interest of time and investigative importance the discussion of liquid phase markers will be restricted to that of polyethylene glycol and the most widely used of soluble dye markers, phenol red. In the comparison of these markers consideration will be given to the reliability of these substances as soluble markers, and to technical considerations for using these substances.

RELIABILITY OF MARKERS

Solubility and Distribution: Several markers have been employed to monitor fluid changes within the gastrointestinal tract. The oldest substance used in this respect is phenol red (phenolsulfonphthalein), which was introduced by Gorham in 1923 (4). Since its introduction, phenol red has been widely applied as a dilution indicator in studies of man and animals. Polyethylene glycol (PEG), on the other hand, was not introduced as a soluble reference marker until some 30 years later (5). It too has been extensively used, and has probably become the marker of choice in the majority of recent studies. Both PEG and phenol red are completely soluble in the liquid phase of gastrointestinal contents, under most dietary regimens. There is, however, one report which indicates PEG as being precipitated out of solution with heather diets. This is reportedly in association with the high levels of tannic acid present in the plant material (6, 7).

Under most conditions PEG, of a molecular weight of 4,000 or less, is highly soluble in water (5, 8, 9). Quantities as great as 500 grams of PEG have been introduced into the ruminant forestomach on consecutive days, without creating supersaturated conditions (5). Polyethylene glycol has also been dissolved in solution at concentrations of 50% (10). High levels of phenol red have not been tested; however, concentrations as high as 10% of the induction solution have been used (11).

While solubility is not a problem, greater difficulty is encountered when seeking uniform distribution of these markers throughout the liquid phase of the gastrointestinal compartment under investigation. Mixing of marker within the liquid phase of gastrointestinal contents is not instantaneous. In a strict biophysical sense, mixing requires turbulent flow (12). Furthermore, neither PEG nor phenol red are explicitly and uniformly distributed only within the liquid phase of ingesta. Soergel (13) has presented an interesting and informative paper on the importance of flow in mixing of markers. The turbulent flow facilitating mixing, as seen in the cardiovascular system, is not generally present within the gastrointestinal tract (Figure 1). In a tubular structure such as the small bowel laminar flow prevails; however, some degree of turbulence is created by the segmental contractions and retrograde movement of ingesta. Thus, several investigators (11, 13-16) working with both phenol red and PEG propose a "mixing segment" prior to the experimental segment, in order to provide for adequate marker distribution. One must realize that this pertains largely to studies of the small bowel. In the ruminant animal with its voluminousforestomach, 30 minutes to 2 hours are generally required for markers to become uniformly distributed within this compartment (9, 10, 17). The complex nature of the gastrointestinal tract of most animals, such as seen within the foregut and/or large bowel of many animals, including man, prohibit rapid mixing of marker and the utilization of a "mixing segment." In such instances continuous infusion and steady state kinetics are generally employed (9, 10, 14, 18, 19).

Marker Recovery: The flow of each phase of gastrointestinal contents can only be accurately assessed if the marker associates exclusively with, and is distributed uniformly throughout one or the other phase of ingesta (20), and can be quantitatively recovered. Unfortunately, neither phenol red nor PEG are exclusively associated with the liquid phase of ingesta. The accuracy of results are subsequently limited as well. Furthermore, adsorption and absorption phenomenon occur for both markers. This, coupled with technical difficulties, contributes to the inconsistencies observed in the use of both markers. In general, comparable studies demonstrate greater erratic recovery of phenol red, than that of PEG (18, 21-23).

With few exceptions (19, 24, 25), polyethylene glycol is 93 to 100%

recoverable in a given experiment. Conversely, reports for the recovery of phenol red average 3 to 8 percentage units below that of PEG, and range from as low as 35% recovery (26). It should again be pointed out that the conditions of the experiment dictate the outcome of the results. Thus, several reports indicate the recovery of phenol red is within the acceptable range, and comparable to that of PEG (18, 27, 28).

Potential adsorption of markers to particulate components of gut contents and/or mucosal surface is of concern for both markers. *In vivo* and *in vitro* rumen studies show PEG associates with both the aqueous and particulate phase of ingesta (10, 29). Labeled polyethylene glycol (^{3}H-PEG) has been shown to adhere to particulate ingesta (17, 29), and estimates range as high as 3% adsorption of this marker (29). However, additional studies demonstrate that adhesion is predominately marker in the aqueous phase which is associated with the so called "wet particulate fraction" (23). The utilization of proper extraction procedures, i.e. washing, centrifugation, etc., greatly reduce or remove the marker-particulate matter association (18, 30, 31). Similarly, phenol red holds an association with the aqueous film layer of particulate matter and the mucosal surface layer. This surface phenomenon was demonstrated by Bloom and co-workers (32) in their experiments showing a 17% loss of phenol red in the pouch of the dog's stomach upon first installation of marker, and only a 5 to 8% loss on subsequent installations, suggesting the saturation of the film layer with marker after first installation. Again, most investigators show improved recovery of phenol red following final wash techniques of the test segment (18, 23, 32). Thus, phenol red and polyethylene glycol appear quite comparable in their resistance to, or association with adsorption properties.

Poor recovery of marker may occur for other reasons, as well. Casein and other proteins are capable of binding phenol red (33), but not PEG (22). Furthermore, polyethylene glycol has proven not to be degraded *in vivo* (5, 34, 35) or *in vitro* (36-38). Unfortunately, the possible destruction of phenol red by *in vivo* or *in vitro* sources has not been investigated.

Absorption properties of the two markers appear to be the area of prominent difference. Both markers are poorly absorbed, but for different reasons. PEG is a hydrophilic-lipophilic polymer of high molecular weight (mol. wt. 3,000 to 4,000 generally used). Although it is sufficiently lipophilic to be absorbed (39) its absorption properties are greatly restricted by its size and shape (5, 40, 41). Phenol red is a triphenylmethane dye of a molecular weight of 1/10 that of PEG (mol. wt. 354), although this molecular weight is thought to be of sufficient size to restrict its passage through the pores of intestinal membranes (18). Phenol red is lipid insoluble, reducing its absorptive abilities, and is hydrophilic and ionized at the normal pH of intestinal contents. Dissociation further reduces its absorptive capabilities and is thought to be the primary reason for its slow absorption (18). Small intestinal absorption rates for phenol red are estimated at 1% per hour in human and rat intestine (42, 43). At a lower pH, however, phenol red is less hydrophilic and is not ionized, making it more susceptible to absorption. Absorption is noticeably greater within the acid environment of stomach contents (26, 32, 44, 45) and in the more acid infusate media (18, 46). Absorption of phenol red from the human stomach has been reported to reach rates of 3.5% per hour (44). PEG is apparently not influenced by the pH of the intestinal or perfusate media.

As one might expect absorption of both markers is generally greater in the disordered gut than in normal tissues (18, 22, 47). It should be noted that PEG, under normal conditions, is absorbed only at trace levels, and that the 5% and 3% PEG absorption reported for the rabbit (48) and the sheep (17), respectively, was obtained from measurements of radio-activity (^{3}H) of labled PEG, rather than a direct measure of the marker itself. Phenol red, on the other

hand, has been recovered in the urine and blood by direct measurements (18, 23, 44-46).

TECHNICAL CONSIDERATIONS

When comparing these markers some emphasis must be placed upon the technical considerations pertaining to their use. Hyden (49) proposed the method for PEG analysis in which the marker develops a turbidity consisting of finely divided oil droplets when associated with trichlorolacetic acid. The turbidity is increased in the presence of barium ions and reaches maximum at 5 minutes. The method described is cumbersome and accuracy limited (effective minimal concentration 0.3 to 0.7 mg/1)(9). While some improvement has been made on the original procedure (10, 26, 50), accuracy remains a problem. Several proposals have been made, and subsequently contradicted, as to the cause(s) contributing to the limited accuracy of the turbidometric method. Smith (51) proposed that unspecified substances within the small intestinal contents interfered with the turbidity of the sample. Corbett and co-workers (37) suggest that losses occur when proteins or sulphate were precipitated in the analytical procedures. However, several investigators observed negligible interference with turbidity (30, 38). Furthermore, neither canine intestinal mucus, human gastric mucus, nor serum proteins interfered with the determination of PEG (38). The presence of phenol red dye in the analytic sample did not distort PEG recovery (22). Much of the controversy has subsequently subsided since the labeling of PEG (^{3}H or ^{14}C) has overcome most of the technical difficulties associated with the marker (17, 29, 52).

Phenol red is determined by colorimetric methods. Spectrophotometric measurements are made at 560 mu, however, several authors propose measuring the optical density at three wavelengths (i.e. 520, 560 & 600 mu) to correct for interference (18, 23, 53). Of noteworthy importance is the fact that the three-wavelength procedure fails when chromogens with absorptive peaks in the 520 to 600 mu range are present (18). French and associates (22) also propose that optical density be measured at 420 mu to correct for bile pigments in the test sample. In general, phenol red (colorimetric) methods are less tedious and more sensitive than that of PEG (turbidometric) methods (23, 54), but not the radioisotopic methods for PEG.

Flow of marker within the tract, and the character of the elution pattern upon marker wash-out are factors of considerable technical importance. Unfortunately, few researchers heed their importance. The classic piston flow is not characteristic of marker movement. While laminar flow is more prevalent, tailing occurs due to diffusion, mixing and retrograde movement of ingesta (13-15) Figure 1). Marker dilution curves thus vary with the time and distance traveled along the gut. Obviously the greater the distance traveled and/or the longer time required to transverse the segment, the more pronounced the tailing (Figure 2). By comparison, short segments and rapid transit curves have a narrow base and a sharp fall in marker concentration once peak concentration is reached (14). Such sharp curves generally yield favorable recovery for both PEG and phenol red. Regardless of the shape of the curve, a marker dilution curve should characteristically exhibit an exponential decline in concentration after peak is reached, provided there is uniform mixing and wash-out (14, 55). Invalid curves can be recognized by the absence of an exponential decline. An abrupt fall-off of the dilution curve suggests piston flow and inadequate mixing (13). On the other hand, the tailing effect, while being characteristic of favorable marker movement, is of prominant importance for both markers, since the concentration of PEG during the extremes of tailing (Figure 2) may fall below detectable levels, leading to poor recovery. And although phenol red's colorimetric method is more sensitive at detecting the lower concentrations, the prolonged exposure associated with tailing contributes to greater absorptive

losses for this marker. In both instances loss of marker leads to an underestimation of the physiological parameter under investigation (10, 13, 22).

Attempts to overcome the problems associated with tailing have involved keeping the dose level high so as to result in a smaller proportion of marker loss. Both markers are, however, osmotically active, PEG in particular (16, 18, 56). Thus, increasing the concentration of PEG disrupts the normal osmotic balance and contributes to the inaccuracy of the biological use for this marker. Hypertonicity of PEG solutions have been shown to inhibit water and electrolyte absorption (16), and for phenol red, hypertonic solution may enhance marker absorption (57).

Neither marker, phenol red or polyethylene glycol is a suitable marker for measuring fat absorption, since both separate into the aqueous phase of ingesta (58, 59).

In summary, we can correctly state that "considerable variability, and some controversy has evolved from the utilization of these markers. Both when considering their individual use, and when making comparisons between the two markers. One can find reports on the comparable and contrasting qualities of phenol red and PEG (13, 15, 18, 21-23, 27, 60), as well as individual studies stating the suitability or inaccuracies of each (5, 10, 13, 16, 29, 32). I would, however, suspect that much of the early work pertaining to appropriate considerations of marker use have been largely overlooked, or perhaps ignored, in some of the more recent research. One can also conclude from the literature search, as has already been correctly stated by Williams (46) and by Miller (23), that the marker "... was a suitable marker for fluid recovery for the purpose of these studies." Or expressed another way, the marker proved satisfactory for the accuracy and the results intended, as set by the expectations of the research design. Furthermore, "... each investigator should validate his own dilution indicator(s) under his particular conditions of study."

PARTICULATE PHASE MARKERS. A variety of markers have been used to estimate transit time of food residues through the gastrointestinal tract (61). Colored dyes and polyethylene tubing are but two such markers used in this respect. Unfortunately, of the many substances tried none appear wholly adequate as particular phase markers, with the possible exception of certain rare-earth elements ·

Contrary to the liquid phase markers, which may be used for studies of intestinal absorption-secretion (18, 27, 44, 62), digesta flow (8, 9, 25, 63, 64), fluid volume of a compartment (10, 14, 64), transit time (11, 48, 63, 65) and digestibility studies (5, 7, 19, 41), polyethylene materials and dye markers of the particulate phase are most frequently used for measurement of digesta movement and fecal appearance. Polyethylene material has, however, become increasingly popular as a particulate phase marker over the past decade (65-68). Although some objections have been expressed concerning its use (20, 69), polyethylene tubing shows several distinct advantages over that of stained food particles. Radio-opaque barium sulphate impregnated polyethylene materials can be detected radiographically in fecal samples, ingesta samples and *in vivo* (66, 67). Furthermore, the marker can be constructed to specified length particles intended for study (65, 67). As such, the weight, length, and specific gravity of particles can be controlled. More importantly, the specific gravity of polyethylene tubing (1.25 to 1.63 {67}) compares favorably to ingesta. Whole feces containing bacteria, epithelial cells, mucus, etc., has a specific gravity of approximately 1 (70). Cellulitic material and undigested fibers within the ingesta have specific gravity between 1.3 and 1.6 (67). Since specific gravity is known to prominently affect digesta passage, polyethylene markers should

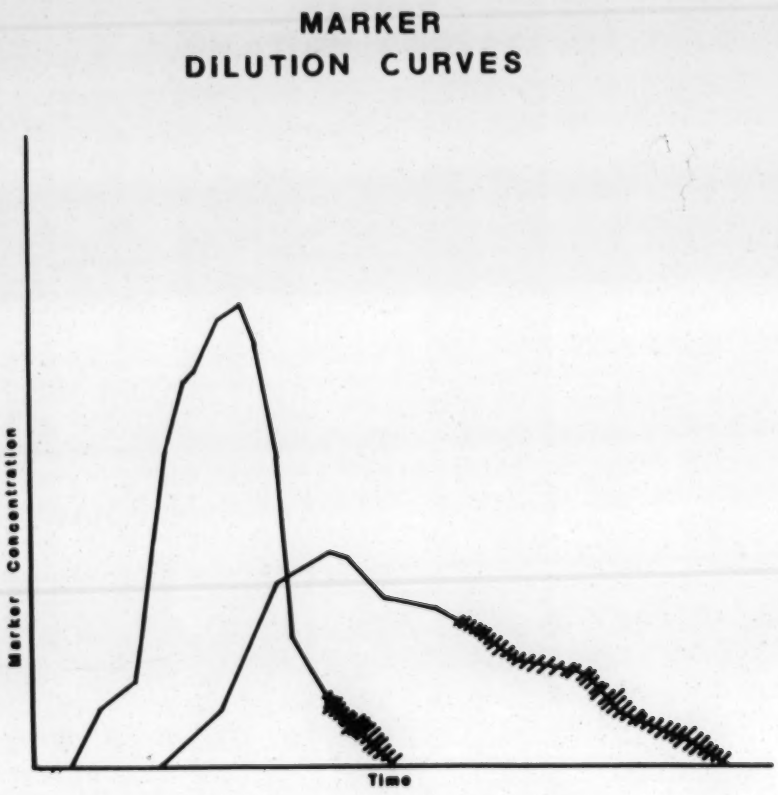

FIG. 2. Schematic representation of dilution curves as influenced by transit time through the intestine. Shading represents area of critical marker recovery. Adapted from Dillard, et al (14).

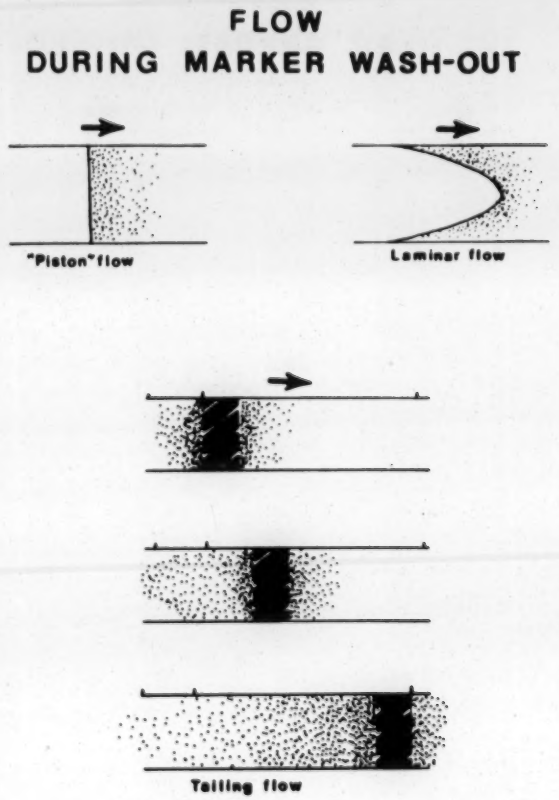

FIG.1. Schematic representation of liquid phase marker flow through the intestinal tract. Adapted from Soergel (13) and Barreiro, et al (77).

behave in a manner similar to dietary residue. Of some concern, however, is the use of hollow tubing polyethylene material (63, 67), since the tubing may trap air and subsequently distort the specific gravity of the intended particle (Stevens, personal communication).

Polyethylene markers are effectively recovered (98 to 100%) with minimal difficulty. Such markers are neither destroyed or altered within the gastrointestinal tract, the exception being rumination and mastication processes (63). Recovery therefore involved washing the sample through a grid (26, 63, 65) or radiographic techniques *in vivo* and/or in the recovered sample (66, 67). Quantity and concentration of marker can be readily determined. Calculated marker pool may then be expressed as the initial appearance, mean transit time, peak marker flow, etc., with considerable accuracy (67, 71). Marking of several meals with polyethylene tubing enables quantative measurements from single stool analysis, suitable for epidemiological studies (71). Polyethylene particles have also been effectively used for digesta flow comparisons across species, and for measurements of ingesta movement between sites within the gastrointestinal tract (26, 63, 65, 68).

Dyes and other staining materials have been used as external markers in an attempt to determine digesta flow (1, 72, 73), as well as fecal markers to trace animal movements (1, 74, 75). While several such dyes have been tested few have proven successful (1). Four dyes show good marking abilities (i.e. methylene blue, crystal violet, basic fuchsin and aniline blue). Five others also stain fecal material, but to a lesser extent (i.e. sulfogene indigo blue, neutral red, safranin O, rose bengal and alizarin red) (1).

Quantitative measurements with stained particles is extremely poor (72), and a 50% recovery of marked particles is considered highly successful (73). Since the loss of stained material is directly related to the length of time such markers are retained within the gastrointestinal tract, estimates of digesta flow, rate of passage, etc., are generally underestimated. The degree of underestimation increases with the prolongation of actual marker flow (73). Such dye markers are difficult to identify within the ingesta or fecal excretion. Indentification is generally limited to visual appraisal (1, 72, 76). These subjective measurements are not only difficult, but extremely variable (72). The single advantage stained food particles have over polyethylene markers is their ability to alter their structure in a manner similar to the dietary residue, as they are passed along the gastrointestinal tract (76). Unfortunately, the stained particle also looses its initial identity and can no longer be described as the movement of a specific particle length (72). Thus, staining of food particles has proven an effective tool in only two respects; (I) in determining the initial fecal appearance of a marked meal, and (II) in monitoring local movements of free-ranging animals.

Regardless of the marker used (polyethylene or dye), or its intended use (liquid or particulate phase), it is important that the researcher understand both the capabilities and limitations of each marker, as well as he understands the biological system under investigation, if meaningful results are to be achieved.

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EFFECT OF COPROPHAGY ON NUTRITION

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ABSTRACT

Coprophagy is practiced to some degree by most animals. Rabbits and rats have been found to recycle at least 40% of their feces. Recent evidence indicates that there is an inverse relationship between the adequacy of the diet in nutrients normally synthesized by the microflora of the intestinal tract and the extent to which coprophagy is practiced. Experimentation has revealed that the rat that does not have direct access to its feces on extrusion from the anus requires a dietary source of thiamin, biotin, pantothenic acid, folic acid, vitamin B-12 and vitamin K. The practice of coprophagy may also influence the digestion and metabolism of the macro-nutrients. Prevention of coprophagy not only helps nutritional deficiencies develop more quickly but may also cause quantitative dietary increases for nutrients. Consideration should be given to the practice of coprophagy when interpreting data obtained from species that practice coprophagy for application with species that do not.

KEY WORDS: Coprophagy prevention, laboratory animals, nutrition

Coprophagy, the feeding or eating of dung or excrement, has been observed in several animals. While it is normal behaviour in such animals as rodents, rabbits, and some primates, it is a symptom of some forms of insanity in man (1) and sometimes a serious problem of the institutionalized retarded (2).

The ingestion of fecal matter by the domestic rabbit was first described by Morot (3), and the extent of this practice was studied by Eden (4) and by Southern (5). Other investigators (6, 7) carried out studies to determine the effect of preventing coprophagy on the rat, and Elvehjem (8) reviewed the possible quantitative and qualitative aspects of recycling the intestinal flora. More recent (9, 10) and similar studies, of shorter duration were carried out to study coprophagy by the rabbit. This review, however, will focus chiefly on studies carried out during the last two decades in order to determine the circumstances under which coprophagy occurs and the nutritional contributions of coprophagy.

THE NATURE AND RATE OF COPROPHAGY

Diet may have an effect on coprophagy, for Michelson (11) stated that coprophagy does not occur when essential nutrients are present in the diet. Other investigators have reported that when all known, required nutrients were consumed, the rabbit (10) and rat (12) recycled from 50 to 80%, and from 42 to 65% of their fecal material, respectively. Although this may have been an over-estimation of the practice of coprophagy, especially since the clearing of the rabbit intestinal tract is a slow process, one report (12)

indicated that most monogastrics have a voracious appetite for feces. In fact, Barnes et al (12) estimated that coprophagy occured at 50% or higher even if the rats were maintained in wire-bottom cages.

In two experiments in which rats were permitted coprophagy after a 24-hr period during which coprophagy was prevented by the use of tail cups, Barnes et al (12) found that 42% and 54% of the feces resulting from the ingestion of a purified diet were recycled. The addition of 2% succinyl sulfathiozole plus 3% ground cellophane to the diet resulted in 52% of the feces being eaten. Likewise, the ingestion of a commercial dog chow increased reingestion of feces to 65%.

In contrast to the possible development of an excessive appetite for feces by the rat after a short coprophagy-prevention period (as above), Barnes et al found that rats had to learn to eat their feces after coprophagy was prevented for a 4-week period (12). Only a small amount of feces was consumed the first day coprophagy was allowed with only 50% of the normal consumption by the seventh day. It took 19 days before coprophagy was fully developed.

While Barnes et al (12-14) found that preventing coprophagy in rats by the use of tail cups resulted in a smaller ingestion of food followed by a smaller excretion of feces than occurred in coprophagic rats, no such effect was found by Giovannetti et al (15) or by Chang and Hegsted (16). In fact, when coprophagy was prevented by placing similar tail cups on rats fed two different diets, the older rats wearing sham tail cups allowing coprophagy, consumed 14% more of the less nutritious wheat diet and excreted 14% more feces than rats without tail cups or with tail cups positioned so as to allow coprophagy (15). Modification in the method of placement of the tail cup from that prescribed by Barnes et al (12) in order to permit less stressful experimental conditions, might account for some of the findings of Giovannetti et al (15). However, evidence from this comparison between fecal excretion of rats wearing anal tail cups and that of rats permitted free access to the anus, as a measure of coprophagy, indicated that coprophagy did not occur to any great extent in the latter study. The feeding of diets adequate in the nutrients normally synthesized by the microflora of the intestinal tract, perhaps reduced the need to practice coprophagy.

COPROPHAGY AND ITS EFFECT ON GROWTH

Contrary to the more recent findings of Giovannetti et al (15), several previous studies (6, 7, 13, 14, 17) provided evidence that rats need to recycle their feces to obtain sufficient nourishment for growth; when tail cups were fitted over the anus, the growth rate was depressed. In fact, it was postulated that when coprophagy is completely prevented there may be a requirement for an unidentified growth factor.

Barnes et al explored such a possibility, using crude materials that had frequently been suggested as sources of unidentified factors for growth (13, 14). They found, however, that this 15 to 25% growth retardation was not prevented by feeding: a) Natural materials such as adenine, adenosine, amylose, ascorbic acid, cellulose, cholesterol, distilled dried solubles, fish solubles, inositol, lactose, liver, mevalonic acid, pancreatin, pepsin, whey, yeast, various proteins, and stock diets. b) Feces or the contents of the cecum of conventional rats by mixing different amounts of dried feces in the diet, suspending it in water and feeding by stomach tube, or placing fresh feces in separate feeding jars. c) Antibiotics, such as penicillin, bacitracin, chlortetracycline, succinyl sulfathiozole, and a mixture of neomycin, colymycin, and chlortetracycline. d) Bacterial cultures of mixed lactobacilli by stomach tube. These results from the feeding of feces and bacterial cultures are not surprising, however, since anaerobic conditions were not maintained. When an effort was made to approximate anaerobiosis such as that occasioned by

the direct consumption of feces from the anus, growth was stimulated in about one-third of the rats studied.

In reviewing these findings, Barnes et al (14) concluded that the efficiency of food utilization for growth was not affected by the prevention of coprophagy, and the growth depression observed when coprophagy was prevented was directly related to the amount and type of food consumed by the rat. Other investigators (15, 16) have also presented evidence of this. In addition, no difference in growth rate was noted by Barnes et al (14) between rats with tail cups and those without tail cups if a poor quality protein or a small quantity of a good-quality protein was fed. Growth depression increased, however, as the protein content was increased in the diets of rats in which coprophagy had been prevented. While this was particularly noticeable as the soy-protein of diets was increased, there was also some growth impairment when high levels of casein were fed. Barnes et al (14) explained these findings by postulating that a metabolic product of protein, such as ammonia that was probably formed in the intestine, was particularly inhibitory or toxic when coprophagy was prevented. Their findings of lower portal vein-ammonia levels in rats prevented from recycling their feces, however, did not support this thesis.

At that time, no mention was made of either a) the possible contribution of methionine, the most limiting amino acid in both casein and soy-protein, by intestinal bacteria, or b) the possible imbalance of amino acids created when these proteins were increased in the diet and the recycling of feces was prevented. How the type of food ingested affects the quantitative and qualitative elaboration of a nutrient or nutrients by the intestinal flora, might account for changes in the rate of growth. Evidence of such will be presented below in dealing with the effect of the prevention of coprophagy on specific nutrients.

COPROPHAGY AND THE EVALUATION OF NUTRIENTS

Laboratory animals that usually practice coprophagy, such as mice and rats, are used to evaluate dietary utilization of feed stuffs for other animal species not practicing coprophagy, as well as potential foods for man. Thus, the effect of coprophagy on the metabolism of essential nutrients and the resulting physical well-being of animals must be considered.

Macromolecules and Their Metabolism (Table 1)

Reports on the utilization of dietary protein, carbohydrate, fat and their hydrolysates are varied (10, 15-31).

1. Protein

a. Rats: Stillings and Hackler (19) found that coprophagy influenced the utilization of protein. In metabolic studies in which 21-day old rats were fed 5 g of eight different 10%-protein diets differing widely in protein quality, prevention of coprophagy decreased: a) The biological value of whole egg, soy protein heated at 121 degrees for 5 minutes and imbalanced and balanced amino acid mixtures. b) Nitrogen absorption of whole egg, soy protein heated at 121 degrees C for 40 and 120 min., possibly because there was an increase in metabolic fecal nitrogen and urinary nitrogen excretion. Also, in a growth study in which rats consumed 10% protein diets ad libitum, recycling of feces permitted greater weight gains and higher values for the protein efficiency ratio of wheat gluten, soy protein heated at 121 degrees C for 120 and 140 min., and the balanced amino acid mixture. These researchers indicated that further investigation was necessary to determine the effect of energy level and feed intake on the extent to which coprophagy is practiced and on the utilization of protein when coprophagy is prevented.

Barnes et al (18) and Giovannetti et al (15), however, found that the "apparent" digestibilities were similar for coprophagic and noncoprophagic rats when various sources of protein were used. The protein sources were: casein (1.665% N and 3.348% N), a source of highly digestible protein, and mixed sources of protein in a commercial chow (3.348% N), an example of poorly digestible protein. Groups of 10, 200 to 250 g were fed for 10 to 14 days before a 4-day fecal collection period in the former instance (18). Wheat (2.45% N) and supplemented-wheat (4.21% N) diets were fed by Giovannetti et al (15) to groups of 12, 80 g rats for 5 days before a 3-day fecal collection period and until rats weighed 300 g before a similar fecal collection period. It was not surprising that "true digestibilities" were also similar, since the feeding of protein-free diets to these rats resulted in similar excretions of metabolic fecal nitrogen whether the rats wore tail cups to cover the anus and prevent coprophagy or whether the rats were without tail cups or wearing tail cups (not covering the anus) that permit coprophagy. Accordingly, the finding of similar urinary nitrogen and endogenous nitrogen values for coprophagy and noncoprophagic rats on the same diet indicated that the utilization and biological values for the protein(s) in both the wheat and supplemented wheat diets were similar (15). In addition, the practice of coprophagy did not affect the availability of the 16 amino acids considered in the study (15).

In considering amino acid metabolism, and using weight as a measure of growth, Chang and Hegsted (16) sought to determine whether the smaller loss of weight caused by a deficiency of lysine, than from a deficiency of certain other dietary amino acids in the diet was a result of ingestion of fecal lysine. They checked the effect of coprophagy on responses resulting from amino acid and protein-deficient diets. When rats were fed a 20% casein diet for four weeks, those wearing tail cups gained 45 g as against 53 g for rats without tail cups. An extension of the study for another 4 weeks resulted in mean total weight losses of 8, 13, 60, and 59 g when rats with tail cups were fed 2.2% lactalbumin, 1.4% of a complete essential-amino-acid mixture without lysine, 1.9% of a complete essential-amino-acid mixture without threonine, and no protein (or amino acids), respectively. Correspondingly, rats without tail cups lost 4, 8, 53, and 56 g. Rats fed the threonine- and protein-free diets lost weight rapidly, whereas those fed the lysine-free diet lost weight slowly whether or not coprophagy was prevented. This smaller weight loss observed in rats fed lysine-free diets was not due to the availability of lysine from the feces.

In still another specific example of how coprophagy affected the protein metabolism of the rat, Barnes et al (21, 22) determined the effect of coprophagy on the stimulation of growth when unheated soybean diets were supplemented with penicillin, methionine, and cystine. When rats were fed semi-purified diets containing whole soybeans at the 10% protein level, feeding penicillin increased trypsin excretion three fold to represent one half the total cystine in the feces (21). This was explained on the basis of the increased production of trypsin in response to ingestion of unheated soybean being protected, by the use of antibiotics, from bacterial proteases, and then excreted in the feces. Thus the recycling of feces was thought to contribute to the cystine requirement for the synthesis of these pancreatic enzymes. While prevention of coprophagy did not affect the normal stimulation of growth when 25% unheated soybean diets were supplemented with cystine and 50% unheated soybean diets were supplemented with cystine or methionine, the 25% unheated soybean diet fed rats did not benefit by the addition of methionine. How methionine supplementation exerts this beneficial effect was not understood, but the authors suggested that there was an impaired methionine metabolism when coprophagy was prevented. In other words, coprophagy was necessary for the conversion of methionine to cystine if growth stimulation was to result when the trypsin-inhibiting unheated soybean diets were consumed.

In order to understand the mechanism of action in the pancreas when

the trypsin inhibitor of raw soybean was fed to rats, Kwong and Barnes (23) used ^{35}S-labelled cystine and ^{35}S-labelled methionine. They found an increased uptake of ^{35}S-cystine for the synthesis of pancreatic protein and increased transsulfuration of methionine sulfur from methionine to cystine whether the rats were fed a diet containing raw soybean or one containing casein, with 50 mg of crystalline trypsin being administered by gavage. When coprophagy was prevented, transsulfuration of ^{35}S-methionine to ^{35}S-cystine and the uptake of ^{35}S-cystine for the synthesis of exocrine protein were blocked.

b. Rabbits: Using roughage or purified diets (25% casein), Thacker and Brandt (10) fed three groups of four 14 week old rabbits (two in stanchions) a constant level of feed intake for 10 days while collecting fecal and urine samples. They found that the practice of coprophagy allowed for an increase in the "apparent" digestibilities of all nutrients when animals were fed purified diets but only in dry matter, protein and ash for the roughage diet. There was also greater retention of nitrogen by rabbits practicing coprophagy. While there was similarity in the composition of the hard feces from both groups of rabbits fed roughage, there was considerable difference in the content of protein, lignin, cellulose and other carbohydrates between the hard and soft feces when coprophagy was prevented. The dry matter resulting from the combination of hard and soft feces of rabbits fed purified diets during the stanchioned period, however, contained more protein and other carbohydrates and less lignin and cellulose than from the feces excreted during the normal period. Apparently, the type of diet consumed and the physiology peculiar to the rabbit, as well as the type of restraint used to prevent coprophagy, all influenced the digestive process in the rabbit. In a study similar to that of Thacker and Brandt, Stephens (24) found that coprophagy increased the digestibility of N in the diet.

By the use of germ-free rabbits that did not consume their soft feces. Yoshida et al (25) showed that the differences noted in the chemical composition of hard and soft feces are not caused by the stress from collaring. Comparison of the amino acid composition of the cecal contents with the hard and soft feces of collared, conventional (CV) rabbits and germ-free (GF) rabbits revealed that the feces of CV rabbits contained more total amino acids per gram of nitrogen than that of GF rabbits and that the cecal contents and feces of CV rabbits contained a higher percentage of essential amino acids than did the samples from GF rabbits. Thus, the nutritional contribution of the intestinal microflora and the benefits of coprophagy to the rabbit are indicated by this study.

c. Guinea-pigs: The findings of Hintz (27) were similar to those of Yoshida (25). When five male guinea-pigs, weighing 320 to 400 g, were fed peletts of alfalfa meal and those weighing 200 to 300 g were fed 5 g of semi-purified diet for 10 days before and during a 7 day collection period, the prevention of coprophagy reduce the "apparent" digestibility of crude protein ($P < 0.01$) in both types of diet.

2. Carbohydrates

Little attention has been given to the effect of carbohydrate and its metabolism except in connection with the feeding of rats (17), rabbits (10, 24) and primates (20). There was little difference in the "apparent" digestibility of lignin, cellulose, and other carbohydrate whether or not coprophagy was practiced or prevented when the roughage diet was fed but the practice of coprophagy greatly enhanced the digestibility of cellulose when a purified diet was fed (10). The dry matter excreted when coprophagy was prevented contained less cellulose and lignin and more of other carbohydrates than the dry matter excreted when coprophagy was allowed. Upon examination of the cecal and intest-

TABLE 1

Summary of the Effect of Coprophagy Prevention on the Metabolism of Macro-nutrients, according to Species

Nutrient	Effect of Coprophagy Prevention	Species	Reference
Proteins	Reduced nitrogen absorption and biological value to approximately 50% of diets tested.	Rat	19
	Increased metabolic fecal nitrogen and urinary nitrogen excretion of diets tested.	Rat	19
	Reduced weight gains and protein efficiency ratios to 50% of diets tested.	Rat	19
	None for fecal nitrogen, "apparent" or true digestibilities of protein tested.	Rat	15, 18
	None for urinary nitrogen, metabolic fecal nitrogen, endogenous nitrogen, and biological values of protein tested.	Rat	15
	None for the availability of amino acids from the proteins of wheat.	Rat	15
	None for the availability of lysine and threonine when lysine- and thronine-free diets tested.	Rat	16
	Impaired methionine metabolism when trypsin-inhibitor present in diet.	Rat	21-23
	Reduced the "apparent" digestibility and nitrogen retention of protein.	Rabbit	10,24
	Difference in protein content of feces that are hard, soft, or both.	Rabbit	10
	Lower content of essential amino acids per gram of nitrogen.	Rabbit	25
	Reduced the "apparent" digestibility.	Guinea-pig	26
Carbo-hydrates	None for "apparent" digestibility of lignin, cellulose, and other carbohydrates in roughage diet.	Rabbit	10,24
	Reduced the digestibility of cellulose in purified diet.	Rabbit	10
	Reduced cellulose and lignin content but increased other carbohydrates in the dry matter of excreta.	Rabbit	10
	Lower "apparent" digestibility of acid-detergent fraction	Rat	17
	Reduced fiber content of feces.	Guinea-pig	26
Lipids	None for digestibility of fat.	Rat	18
	Reduced the availability of essential fatty acids.	Rat	27
	Reduced the degradation of choline, deposition of fat in the liver, and levels of acetylcholine in the kidney, brain and small intestine.	Rat	29
	Reduced tripalimitin and tristearin but no effect on triolein.	Rat	31

inal contents which are ingested by the Lépilémur under normal conditions, Hladik et al (20) concluded that the very diversified bacterial flora observed in the cecum were responsible for the consistent degradation of the celluloses and the hemicelluloses while in that area of the intestinal tract.

When coprophagy was prevented, the "apparent" digestibility of the acid-detergent fraction was lowered in the rat (17) and the guinea-pig (27).

3. Lipids

While the digestibility of fat was not altered by the prevention of coprophagy when low-protein, normal-protein or commercial chow diets were fed to rats, Barnes et al (18) suggested there may be quantitative differences if there is microbiological synthesis of essential fatty acids in the large intestine. If such is the case, they are not absorbed in this area, for when essential fatty acid deficient diets were fed, noncoprophagic rats developed signs of essential fatty acid deficiency more quickly than coprophagic rats (27). More recent studies (28-30) have shown that the deposition of fat in the liver may be affected by coprophagy. Kwong and Barnes (29) found that dietary supplements of chlortetracycline or pthalylsulfathiazole or the prevention of coprophagy reduced the degradation of choline to trimethylamine by intestinal microflora. Thus it seems that the prevention of coprophagy spares choline, reduces fat accumulation in the liver, and maintains near-normal levels of acetylcholine in the kidney, brain and small intestine.

Monitoring the fatty acid content of the digestive tract in four Lépilémurs, Hladik et al (20) found that while there was similarity between the fatty acid content of the food and that of samples from the stomach 7 hr after ingestion of the food, cecal samples no longer contained linoleic and linolenic acids, but contained a large quantity of short-chained fatty acids provided by bacterial lipids. Sixteen hours after the ingestion of food, however, the fatty acid composition of the stomach contents approached that of cecal contents found previously, but with a smaller short-chained fatty acid content. This suggested that the bacterial fatty acids were partially absorbed upon ingestion of fecal material.

When triglycerides were fed at two different levels as 5 or 25% triolein, and tripalmitin or tristearin fed as 7 g of diet to 180 male, weanling rats that had been fitted the previous week with tail cups or with sham tail cups allowing coprophagy, fecal samples were saved for seven days before the rats were sacrificed (31). Prevention of coprophagy resulted in a marked reduction ($P < 0.01$) in the apparent digestibilities of tripalmitin and tristearin, and consequently, in a lesser weight gain in these rats. Triolein utilization was not affected.

Micromolecules and Their Metabolism (Table 2)

Other investigators (5, 7, 12, 26, 31-43) have dealt with the vitamins and minerals known to be synthesized in the intestines of some animals. Since absorption may not occur in the same area of the intestine as the synthesis, the practice of coprophagy may play some part in making such nutrients available to an animal.

1. Vitamins

From the chronological order in which these experiments are cited below it seems that the effect of coprophagy on nutrients was determined by a) prevention, using tail cups designed by Barnes et al (12) in the earlier studies, and b) by the use of antibiotics to control intestinal, bacterial colonies in later studies.

a. Vitamin B-12: When Barnes and Fiala (32) fed weanling rats a highly purified vitamin B-12 deficient diet, no evidence of deficiency was observed during the rapid growth period unless coprophagy was prevented. Such a growth depression only 1 or 2 weeks after the rats consumed the deficient diet indicated that it was not difficult to deplete vitamin B-12 stores during a rapid growth period. While feeding feces to the vitamin B-12 deficient, noncoprophagic rats returned their growth rate to the level of noncoprophagic rats fed the commercial chow diet, modifying the fat content of the diet aggravated or lessened growth when low- and high-fat diets were fed, respectively. Likewise the addition of succinyl sulfathiazole to the diet aggravated the deficiency beyond that experienced when rats were fed low-fat diets or low-fat, vitamin B-12 deficient diets for 7 weeks (32).

Further investigation by Barnes et al (33) also revealed that when coprophagy was prevented, the sparing effects of penicillin, aureomycin and bacitracin for vitamin B-12 as determined by growth assay, were not observed. A study by Armstrong and Curnow (34), however, showed that coprophagy was not necessary to the vitamin B-12 sparing action of antibiotics, as determined by total urinary ether soluble acid, methyl malonic acid and vitamin B-12 assays on liver and intestinal tract.

b. Biotin: Barki et al (7) and Barnes et al (35) noted a depressed growth rate in weanling rats fed a biotin deficient diet when coprophagy was totally prevented. The feeding of unsaturated fats aggravated this condition of mild biotin deficiency resulting from the non-availability of the biotin newly synthesized by intestinal bacteria, and produced skin lesions such as alopecia, dermatitis and encrusted eyes by the time the rats were 15 weeks of age. Similar results were obtained in half the time by the binding of the intestinal biotin when 25% of the diet was composed of raw egg white.

There was no consistent change in total liver fat and cholesterol when biotin deficient diets were fed to coprophagic and noncoprophagic rats but storage of liver cholesterol was increased in the mildly biotin deficient, noncoprophagic rats when 1% cholesterol was added to the diet. Serum cholesterol levels increased more, however, in the coprophagic rats fed a biotin free diet except when cholesterol was added; then, the serum cholesterol of noncoprophagic rats was higher. Thus, the biotin deficiency, created in rats not recycling the intestinally synthesized biotin and aggravated by the addition of cholesterol to the diet, interfered with cholesterol metabolism.

c. Vitamin K: Using prothrombin time as the sole criterion of deficiency, Barnes et al (36) found great variability when vitamin K deficient diets were fed to rats not permitted the practice of coprophagy. Since a deficiency of vitamin K may affect proconvertin, plasma thromboplastin and prothrombin, the authors suggested that a one-stage prothrombin time determination does not adequately measure the entire metabolic effect. While rats died when prothrombin times were only slightly above normal, the greatest mortalility rate was associated with the highest prothrombin times.

Barnes et al (36) also suggested that the peaked increase in prothrombin time at 3 weeks post-weaning, followed by a fall and then the highest peaking increase at 6 weeks, might be the result of changes in the microbial population. Coprophagy, however, does not affect the colonic flora, according to Schulze and Haenel even though the prevention of coprophagy results in the development of vitamin deficiency symptoms with diets deficient in vitamin K (37).

d. Thiamine/Riboflavin: Mameesh et al (38) fed a thiamine limiting and a thiamine adequate diet (0.5 and 10 µg thiamin.HCl per gram of diet) to male weanling rats for 4 weeks in two different experiments. The weight gained by those consuming the thiamine limiting diet and allowed coprophagy

was 46 ± 8.4 g and 39 ± 2.2 g as compared with 24 ± 4.2 g and 19 ± 4.3 g for noncoprophagic rats. The weight gain for rats on the thiamine adequate diet and practicing coprophagy was 113 ± 3.8 g compared with 142 ± 4.8 g and 125 ± 6.4 g for rats on the same diet not permitted coprophagy. Enhancing the weight gain of rats permitted coprophagy only when 50 mg of crystalline penicillin were added to the diet suggested to Mameesh et al that penicillin stimulated production rather than absorption of thiamine synthesized by the intestinal microflora. In other words, the thiamine produced was not available to the rat except by the recycling of the feces.

A series of experiments by Barnes et al (39) confirmed the hypothesis of Mameesh et al (38) that penicillin in the diet increased the amount of thiamine synthesized in the large intestine (and thus excreted in the feces) and thiamine synthesized in the intestinal tract was absorbed only after excretion in the feces and the ingestion of those feces. As thiamine intake by stomach tube was increased, so was increased the food intake, weight gain (including liver weight), amount of feces excreted, and the amount of thiamine in the liver whether penicillin was given or not. Fecal thiamine decreased, however, as dietary thiamine was increased and a penicillin-free diet fed. With the addition of penicillin, fecal thiamine concentration increased.

The influence of dietary carbohydrate on refection of thiamine and riboflavin was studied by Guerrant et al (17). When coprophagy was prevented in rats fed thiamine and riboflavin free diets, refection of these two vitamins did not occur regardless of the source of carbohydrate. Refection did take place, however, only when dietary raw-starch based diets were used and coprophagy was allowed. Employment of sensitive biochemical tests of riboflavin status such as measurements of NAD(P)H_2: glutathione oxidoreductase (EC 1.6.4.2), succinate: oxidoreductase (EC 1.3.99.1), and NADH: oxidoreductase (EC 1.6.99.3) indicated that marginal refection of riboflavin through coprophagy was possible when a sucrose-based diet was fed to rats (39).

e. Pantothentic acid: Feeding pantothenate limiting and pantothenate adequate diets (1 and 20 µg calcium pantothenate, respectively, per gram of diet) to male weanling rats for 5 weeks in two different experiments brought these results: The pantothenate limiting diet-fed rats allowed coprophagy gained 69 ± 9.1 g and 63 ± 8.2 g as compared with 55 ± 5.3 g and 44 ± 7.4 g when coprophagy was prevented. The pantothenate adequate diet-fed rats practicing coprophagy gained 206 ± 7.0 g and 210 ± 12.3 g, compared to 207 ± 9.3 g and 164 ± 5.3 g for noncoprophagic rats (38). The addition of 100 mg of oxytetracycline to each kilogram of diet stimulated the growth of rats fed the pantothenate limiting diet but was without effect when the vitamin was adequately supplied, whether or not coprophagy was allowed. This indicated that the pantothenate sparing action of oxytetracycline might result from increased synthesis by the intestinal microflora and secretion extracellularily into the medium for ready absorption.

Studies by Daft et al (41) to determine the sparing effect of another antibiotic (penicillin) and ascrobic acid on pantothenate brought results at variance with Mameesh et al (38). While the growth of conventional rats fed a pantothenate free diet were protected by the addition of penicillin and ascorbic acid, germ-free rats on similar diets failed to respond to such additions (41). When experimental conditions were made to simulate those used by Mameesh et al by using conventional rats and preventing coprophagy by the use of tail cups, growth did not improve by the addition of penicillin and ascorbic acid to pantothenate free diets.

f. Folic acid: Using folate-free diets, Daft et al (41) found that germ-free rats displayed slow growth, leukopenia, granulocytopenia, anemia, and excreted formimino glutamic acid in the urine while conventional rats,

except in one instance, were free of these folate-deficiency signs. When six of eight germ-free rats were mono-contaminated with Escherichia coli and half of the six were cupped to prevent coprophagy, the germ-free rats died at 65 and 72 days, the mono-infected and non-cupped animals showed the best growth and highest levels of folic acid in the liver and urine, and the mono-infected and cupped animals showed intermediate values indicating that some folate was available to the cupped rat even though coprophagy was not practiced. A greater growth response, even in noncoprophagic rats, might have been found had conventional or germ-free rats been conventionalized by poly- rather than by mono- infection.

2. Minerals

Little is known of the effect of coprophagy on mineral metabolism for few experiments have been designed to determine if the intestinal microflora may alter minerals in some way to make them more or less biologically available when recycled.

a. Iron: An experiment (42) was designed to determine if the prevention of coprophagy by the use of tail cups affected the absorption of iron. Male rats weighing from 265 to 305 g after an adjustment period of 1 week to a new diet and environment, were fitted with tail cups and sham tail cups. After 24 hr without water and food, each animal was given 0.23 μCi of ^{59}ferrous sulfate by gastric instillation. Each dose was dissolved in 1 ml of distilled water to which 5.0 mg vitamin C and 0.24 mg ferrous sulfate as carrier iron had been added. There was no difference in whole-body counts between the rats wearing coprophagy-preventing tail cups and those wearing coprophagy-permitting tail cups while the greater iron absorption of the control group had borderline significance. Neither was there any difference in hematocrits between the three groups. These researchers concluded that coprophagy did not play an important role in short-term studies such as this in which rats were kept on wire screen flooring and handled daily. Hintz, however, found that the prevention of coprophagy of the guinea pig decreased the availability of iron (26).

b. Calcium, phosphorous, magnesium: When calcium and phosphorous were fed at three different levels (0.08, 0.50 and 2.06% calcium carbonate plus 0.15, 0.58 and 1.67% potassium monobasic phosphate) with two levels of triglycerides (5 to 25% triolein, tripalmitin, or tristearin) as described above, prevention of coprophagy resulted in a marked reduction ($P<0.01$) in the apparent digestibility of calcium, phosphorous, and magnesium at any of the three levels of calcium and phosphorous tested (31). The noted improvement in the digestibility of these minerals as the result of coprophagy did not, however, influence their retention in the femur, the total ash in the femur, or the moisture-free, fat-free weight of the femur.

Hendrick and Mahoney (43) found that the prevention of coprophagy did influence the bone concentration of calcium and phosphorous. When sodium phytate replaced 0, 20 or 40% of total dietary phosphorous in a basal, casein diet, there was a significant increase in the concentration of phytate and calcium in the feces of collared rats on all diets as well as a significant reduction of bone calcium and phosphorous in rats receiving a 40% phytin phosphorous diet when coprophagy was prevented.

c. Others: In guinea pig experiments, Hintz (26) found a significant increase in the amount of these minerals as well as potassium, zinc, manganese and copper excreted in the feces. The addition of 250 ppM molybenum to the diet appeared to decrease the amount of all minerals except zinc present

TABLE 2

Summary of the Effect of Coprophagy Prevention on the Metabolism of Micro-nutrients, according to Species

Nutrients	Effect of Coprophagy Prevention	Species	Reference
Vitamins			
B-12	Depleted vitamin B-12 stores quickly when vitamin B-12 defficient diet fed.	Rat	32
Biotin	Reduced availability of biotin and increased serum cholesterol when biotin deficient diet fed.	Rat	7,35
K	Affected prothrombin time when vitamin K-free diet fed.	Rat	36,37
Thiamine/ Riboflavin	Reduced refection and availability of thiamine when thiamine limiting diet fed.	Rat	17,38-40
Pantothenic acid	Reduced availability of pantothenic acid in conventional and germ-free rats fed pantothenate limiting diets.	Rat	38,41
Folic acid	Deficiency signs in germ-free rats fed folate-free diets.	Rat	41
Minerals			
Iron	None for absorption.	Rat	42
	Decreased the availability of iron.	Guinea-pig	26
Calcium, phosphorous, magnesium	Reduced "apparent" digestibility of calcium, phosphorous and magnesium at three different dietary levels of calcium and phosphorous.	Rat	31
	None for retention of these minerals in the femur.	Rat	31
	Increased excretion of calcium and phosphorous regardless of the level of phytate replacement of dietary phosphorous.	Rat	43
	Reduction in bone calcium and phosphorous when phytate replaced 40% dietary phosphorous.	Rat	43
Others	Increased excretion of potassium, zinc, manganese and copper.	Guinea-pig	26

in the feces when coprophagy was prevented but not when coprophagy was allowed.

With coprophagy prevention, development of nutritional deficiencies and quantitative dietary increases for some minerals and vitamins, as well as for the essential fatty acids, may result from a change in the intestinal microflora; Gustafsson and Fitzgerald (44) found a reduction of lactobacilli. Consideration should be given not only to the reduced intake of the micronutrients usually found in the feces but also to the possibility of variations in the level of nutrients secreted or depleted by the altered intestinal microflora when coprophagy is prevented. In addition, since many micronutrients are poorly absorbed in the large intestine, their return to the upper intestinal tract may be critical when there is marginal dietary intake and/or biological availability.

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CONFERENCE SUMMARY

International Workshop on Nutrition and the Development of the Child

The workshop was organized by Professors C.V. Ramakrishnan and R. Rajalakshmi, Department of Biochemistry, M.S. University of Baroda and held January 8-15, 1982 in Baroda, India. The objectives of the workshop were to:

(i) identify how nutrition and environment interact to stimulate or constrain the physical and mental development of infants and children;

(ii) to assess the prevalence and impact of these factors in regions which illustrate the problem in developing countries,

(iii) to illustrate how social actions within the existing framework might help improve development and performance and reduce the constraints imposed on children and their families at risk for malnutrition, and

(iv) to indicate research priorities based on identification of major problems and how specific types of research being carried out in underdeveloped as well as highly developed countries might be relevant to and adapted for use in the developing world.

The workshop accomplished these objectives to a considerable degree. 65 delegates participated from 17 countries, developed and developing, and representing a broad range of social, economic and political structures. Particularly noteworthy was the absence of arrogance by workers from privileged areas and of defensiveness by those from less fortunate countries. Of particular importance, new channels of communication were opened and new contacts and friendships were established among the representatives of many countries. Names on publications, known to many, were transformed into people.

Nutritional and physiological status during pregnancy

Nutritional needs during pregnancy must consider the physiological requirements when conception occurs before adolescent changes have been completed. Studies are needed: on physiological adaptations during pregnancy which are known to occur in the case of several nutrients such as protein, calcium and folate; on interaction of nutritional and non-nutritional factors (e.g. physical labor, poverty, infections, etc.); and on food and nutrient intakes associated with satisfactory fetal development. Such data must be obtained before dietary requirements during pregnancy can be based on rational and empirical considerations rather than on the concensus of guess work by experts. Indices for unsatisfactory fetal development are essential if interventions during pregnancy are to be based on real need. Studies also are needed to provide data on birth weights, taking into account the duration of pregnancy and the differential expectations for fetal growth in different mothers from different societies. Weight gain during pregnancy correlates with birth

weight, but rather weakly. In fact differences in both weight gains and birth weights occur on similar intakes within any given population. While pregnancy weight gains and birth weights serve as indices of fetal nutrition in the community, their prevention by appropriate intervention measures in the case of individuals at risk needs early detection.

Determinants of fetal development

Fetal malnutrition is an enormous public health problem, particularly in developing countries where 13 million babies may be affected annually. These infants are at risk for poor postnatal physical and mental development. Although the precise type and quantities of nutrients which will benefit mother and fetus most during pregnancy are not known, the workshop presented good evidence that maternal nutrition is related to fetal growth. Among the nutrients, the levels in the blood iron, zinc, and magnesium, blood glucose level, certain aminoacids, cholesterol and some triglycerides were shown to be significantly related to fetal growth. Standards for the growth of the human fetus, based on actual measurements, were presented. The session contributed new concepts. Evidence was presented that the adequacy of intrauterine nourishment and growth of the human fetus should be evaluated on how nearly it attains its expected size, given the duration of gestation, size, race and age of the mother, and the occurrence of infections or other complications during pregnancy. Instead of some arbitrary weight like 2500 gm, babies whose birth weight is more than 1 or 1½ standard deviations below their expected weight may be considered too small. These are the babies at risk for perinatal complications and poor future development risk. Another concept was that the over all balance of nutrients, rather than 1 or 2 nutrient deficiencies is reflected in the maternal nutrient blood level patterns which reach the fetus and affect fetal growth. Finally, evidence was presented to show that it is possible to predict at mid-pregnancy with reasonable accuracy the birth weight the baby is likely to have. This can be done from clinical and biochemical measurements obtained from the mother. Such predictions should permit the identification of those fetuses and mothers most likely to benefit from a nutritional intervention.

Postnatal care and development in relation to gestational age and weight at birth and other factors

The third session was concerned with the biochemical development of the infant after birth and the nutritional implications of this development. The biochemical development of the gastrointestinal tract and its adaptation in terms of the proximate constituents of human milk was discussed. The possibility that certain proteins in milk might be absorbed intact was raised. Biochemical development of the liver was described with particular reference to the metabolism of certain aminoacids, particularly taurine. This amino acid may modulate growth of certain cells and some aspects of neurotransmission. Implications for feeding the low birth weight infant whose biochemical development is insufficient to handle certain amino acids was discussed in view of these developmental features. The case for feeding human milk to preterm infants was emphasized. A multitude of cultural, social and psychological factors superimposed on developmental limitations and contributing to impaired gestational and lactational performance were detailed and their potential effect on later behavioral development was emphasized.

Lactation and milk composition

The main nutritional properties of human milk as compared to cow and buffalo milks were reviewed. Studies showing the biological advantage to the

infant of the various substances present in fresh human milk (e.g. amino acids, immunoglobulins, hormones, etc.) were stressed. Ways were suggested as to how some milk formulas derived from cow's milk can be supplemented and modified to improve their handling by the gastrointestinal tract of the human newborn.

Breast feeding, bottle feeding and weaning practices

The session on breast and bottle feeding practices produced a variety of papers which explored many aspects of the problem. For example, in a rural Hospital and Medical Center nursery in the Philippines the incidence of oral thrush, diarrhea, clinical sepsis and deaths were dramatically reduced among 9886 babies over a period of 4 years when early breast feeding with rooming in were instituted as a policy. Significantly fewer infant infections and lower mortality occured among the breastfed and mixed fed than among the formula fed infants.

Some studies of breast feeding patterns in rural and urban India indicate that breast-feeding is maintained for prolonged periods in rural areas and by the urban poor although the intrusion of bottle-feeding in urban areas to varying degrees is evident.

In Kenya, better nourished mothers produced better milk output than less well nourished mothers, although energy intake in both groups was below recommended standards. More surprising was the finding that although yields were significantly lower in the less nourished group, differences in infant nutrition were relatively small.

The session made the point that perception of weaning practice behaviour requires change from a static, oversimplified value-ridden event to a behaviour which occurs over time. We need to develop methods for observation of the feeding practices of mothers and other caretakers towards the child within a family and community context.

The importance of cultural beliefs, folk practices and traditions for maintaining breast feeding patterns were stressed.

Even in a developed country such as the USA national surveys found a strong inverse relationship between duration of breast feeding and indicators of economic development and modernization. There is a complex interaction between many food and environmental factors and the duration of breast feeding. Many of the factors which limit the duration of breast-feeding appear to be irreversible aspects of the world today.

Even in the USA, high infant mortality and morbidity in a poor, inner city population was associated with only 15% of mothers initiating breast feeding and less than 1% still breast feeding at one month of age. Health workers may be inexperienced regarding breast feeding practices. Primary health care workers, trained in breast feeding management skill can result in more women choosing to breast feed and a larger proportion of infants exclusively maintained on breast feeding rather than supplementing with early formula with resultant better development.

Another study revealed the widespread prevalence of pathogens in samples of water and weaning foods, swabs obtained from mother's nipples, nails, child-'s hands, utensils etc. Further studies are needed to identify pathogens in the feces of breast-fed babies living under adverse types of environmental conditions.

Environmental, hormonal and emotional factors in the development of the infant

In the session on the effects of environmental, hormonal and emotional factors on the development of the child, mother-child interactions, particularly stresses, were shown to cause measurable changes in adrenal hormone levels in the infant monkey. Among the emotional factors, facial expressions of the normal neonate to different taste stimuli may be a clue to the expression of emotions in later life. Studies are needed regarding alterations in taste threshold in malnutrition. A fascinating film was shown illustrating the role played by mother-child interactions on the emotional, cognitive and intellectual development of the child.

Cultural patterns have limited the use of valuable foods for improving nutrition on the one hand. On the other hand loss of cultural wisdom has been responsible for replacing many valuable foods, particularly weaning foods, by non-nutrients such as tea, coffee, and by sugar and fat.

Regarding the development of emotional behaviour, experimental studies with squirrel monkeys indicated that the infant's capacity to cope with stressful situation depends on its achieving a normal relationship with its mother. Malnutrition probably influences this relationship through apathy and lags in the development of the child and may consequently have effects on the child's emotional development. The use of piagetian strategy and task evaluation may help define effectiveness of this psychological development. For example, developmental assessment using modified piagetian techniques indicates that cognitive reasoning and spatial memory may be adversely affected in the malnourished child.

Surveillance of growth and development during childhood

The session on this topic emphasized the importance of surveillance system in detecting deviations from normal growth and development. The system needs to be an integrated one, involving not only collection of growth data, but also its determinants like food and dietary pattern, socio-economic conditions and cultural practices of child rearing and health concepts. How the National Monitoring Bureau established by the Indian Council of Medical Research which with its Central Reference Laboratory at the National Institute of Nutrition, Hyderabad and ten regional units in different states, works to conduct and coordinate collection of data on diet, clinical and anthropometric nutritional status of representative samples of the Indian population was described. Continuous collection of relevant data and their periodical analysis, interpretations and reporting is basic to the concept of surveillance. The constraints and pitfalls in the organization of that net work for data collection was described. The selection of suitable indicators, and fixing of 'cut-off' levels for the indicators was deemed particularly important. The practical problems in the organisation of a comprehensive action program for mother and child in squatter slums was discussed. How this might serve not only as a tool for mother's education was described. Longitudinal growth studies are particularly useful for assessing the impact of nutritional and other factors of development.

A rational method of fixing 'cut-off' levels for various indicators, based on functional criteria, needs to be developed, since growth indices such as weight, height, arm circumference and different ratios based on them have limitations.

Ecological, physiological and biochemical correlates of severe malnutrition in children

The prevalence of severe malnutrition in the poor areas of the world is found to persist but the proportion of marasmic cases is found to increase and this shift is associated with the earlier introduction of the bottle. The biochemical profiles in severe PCM vary mainly with regard to serum proteins which are sometimes low in marasmus, and with regard to serum vitamin A and magnesium which are affected in some regions but not in others.

Severe malnutrition is also associated with gross skeletal retardation and abnormal features in bone morphology as assessed by radiological examination.

It is not always possible to identify the correlates of malnutrition in terms of family size, birth order, birth spacing, etc.

The prevention of childhood malnutrition is essentially a political problem in that governments by socio-economic and idealogical changes could wipe out the condition. Until then, the Ministers of Health and nutritionists must continue to identify, quantify and treat the affected children.

Health workers and nutritionists, by working with women's organisations, rural development and education agencies, could increase an awareness of severe malnutrition in a community, identify the factors that make for malnutrition and by cooperation and coordination, work for its prevention.

Nutrition and immunology

Severely malnourished children have impaired immune responses on the basis of several criteria such as delayed hypersensitivity, decrease in the number of circulating T lymphocytes, and alteration in the number of T cell subsets, reduced number of intraepithelial lymphocytes, decreased levels of secretory IgA , antibody affinity, opsonization, bactericidal activity of polymorphonuclear leukocytes, serum complement components, production of lysozyme and interferon. Secretory antibody response to measles and polio vaccines may be depressed in malnourished children. In small-for-gestational age infants, persisting deficits may be found in cell-mediated immunity. The immunocompetence of the child may serve as an index of nutritional status.

Impact of malnutrition on the development of the nervous system in man

Severe malnutrition not only affects immunocompetence, but also impairs the myelination of nerve fibers and damages various regions of brain and spinal cord. This causes neurological changes in children with kwashiorkor. Children with the most severe type of PEM also may have reduced brain weight and cerebellar development. Maternal malnutrition causes relatively less severe effects on brain size as evidenced by reduced head circumferences. Intellectual development of children with severe malnutrition may be impaired compared to their siblings and children from similar socio-economic groups. In children with severe PEM, there is significantly lower oxygen consumption inspite of much greater consumption of glucose in the brain. The rhesus monkey is a good model for such studies. Brain DNA and DNAses are affected in animals with malnutrition and improve with rehabilitation. Apparently, the white matter of the brain was more susceptible than gray matter. Clinically in children with marasmus and kwashiorkor, characteristic EEG changes occur.

Animal studies on the development of the nervous system

Observations on animal models have shown that undernutrition results in a retardation of the biochemical and structural development of the brain.

Electrophysiological studies have indicated that in addition to retarded maturation, highly abnormal electrical activity is also detectable in the brain of undernourished developing rats. Besides overall retardation it is probably more telling that deviations in the normal pattern of brain development are also observed, e.g. affecting the coordinated development of transmitting systems. Furthermore, certain changes are not completely corrected by nutritional rehabilitation and it would appear that especially oligodendroglia are sensitive to nutritional deficiencies.

Even in animal experiments, undernutrition is associated with social and psychological factors which can affect brain development. Thus the observations are consistant with the view that plans for positive rehabilitation should take full advantage of the plasticity of the central nervous system.

Animal studies on nutrition and behaviour

In the human situation it is very difficult to disentangle the effects of undernutrition from those of other potentially harmful influences associated with poverty. In experiments with laboratory animals it is more possible to standardise environmental factors other than nutrition.

Undernourished infant animals showed impaired learning and memory with nutritional rehabilitation. Some but not complete restoration of function occurs with effects persisting in aspects of learning, social including maternal behaviour, and capacity to cope with stress. These findings emphasis how imperative it is to prevent, not just to cure, malnutrition.

Behavioural development in relation to nutritional and non-nutritional factors and prevention of retardation of psychological development in undernourished children

One of the major things recognised in several discussions was the need to consider the interactions between aspects of the child in social environment and maternal status as they affect significantly behavioural development. Particular attention needs to be directed to specific aspects of the home enviroment, particularly the pattern of mother-child interactions involved both in feeding and child care generally.

Several empirical papers reported relationships between malnutrition and mental development, but these showed different aspects of psychological functioning. Also, some malnourished children's performance seems unaffected by malnutrition.

Intervention strategies must be sensitively adapted to cultural characteristics and should be tailored to meet the needs of particular communities and of meaningful sub-groups of families within the community. Finally, active community involvement is an essential ingredient of successful intervention, which should be comprehensive rather than unidimensional in nature.

Nutritional intervention measures during pregnancy and early childhood

In addition to the 13 million babies born with undernutrition, at least 5% of infants in developing countries suffer from marasmus or kwashiorkor, and probably more from vitamin A deficiency. From birth to death, the majority of people in the developing world would never see a professional health worker. Yet nutrition intervention programmes, if they are to be effective, must be targeted to reach the most needy, to provide needed nutrients in the form of locally available foods, where possible, and achieve their goals through

participation by workers drawn from the community and trained to their tasks. The variables which are likely to be most effective in identification of target groups must be clearly defined. To determine whether a nutritional intervention is achieving its goals requires predetermined specific objectives and early repeated critical evaluations of whether or not these objectives are being attained by each segment of the programme or else much time, effort and money can be wasted before faults are detected.

Several features of nutrition intervention are clear, e.g. attention must also be given to improving and protecting water supplies, sanitation and health. It was emphasized that extreme poverty, limited to little or no education, social and political circumstances are responsible for malnutrition. These must be reorganized to help the impoverished, or nutritional interventions will be ineffective in the long run. The existing framework which provoked malnutrition does not suffice, and will not do. The major advances in the field of nutrition during the last 25 years were enumerated, such as the formulation of more realistic nutritional allowances from the point of view of theoretical and practical considerations; the evolution of important intervention measures based on either specific nutrients, such as iron, folate or vitamin A or supplementary feeding programmes, with the shift in emphasis from high protein foods to locally available foods; the need for caution against going too far to the other extreme and the danger of deemphasizing critical nutrients, while concentrating on food energy; and the need for, and value of improving school health services and the critical role school boys and girls can play as agents of social change in their own families and communities.

The workshop was sponsored by COSTED, UNICEF, IBRO-UNESCO, Indian Council of Medical Research and University Grants Commission. The texts of the main talks will be published in due course.

J. METCOFF

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COUNTRY REPORTS

AUSTRALIA

Human Nutrition in Australia: A Personal Comment

After many years of neglect, the discipline of human nutrition is "on the move" in Australia. In the past we have had outstanding contributors, such as the late Professor Eric Underwood in the trace element field and Dr. F.W. Clements in the area of growth and nutrition in childhood. Other individuals, including clinicians and clinical researchers, have made important contributions to related topics such as cardiovascular physiology and disease. But human nutrition has generally lagged behind as a scientific discipline in this country and its professional image has been low.

Recent developments are helping to change this. Departments of Human Nutrition have been established in Deakin University (Geelong, Victoria) and Sydney University. The nation's foremost Government-sponsored scientific institution, the Commonwealth Scientific and Industrial Research Organization (CSIRO) has now established a Division of Human Nutrition in Adelaide (South Australia) and the Federal Government has shown interest in other ways. It supports a small Nutrition Section in the Health Department in the national capital (Canberra) which publishes a regular magazine, "Journal of Food and Nutrition", which is of interest mostly to nutritionists and dietitians. The community and Government have also acknowledged that nutritional disorders are common despite, or perhaps because of, our affluence and this has prompted the Government to adopt a series of simple, sensible guidelines to help overcome this. The National Heart Foundation has been prominent in the campaign against cardiovascular disease and the current decline in its incidence is encouraging.

The Nutrition Society of Australia has now been active for several years. One of its main aims is increased understanding, collaboration and communication between workers in human, veterinary, agricultural, industrial and other nutritional areas. The importance of primary production in Australia has been such that nutritionists in non-human fields have been extremely active and in many instances have achieved international eminence. The value of collaboration between nutritionists in human and non-human fields is exemplified by the discovery that Menke's Kinky hair disease is due to a defect in intestinal absorption of copper. The apparent similarity of the abnormal morphology of the human hairs to the abnormalities in the fleece of copper-deficient sheep with "sway-back" disease led investigators on the right path to detect the abnormality in children with Menke's disease. The Nutrition Society has regional groups which meet regularly and the annual national conference is published in its annual *Proceedings* (ISSN-0314-1004). The present Secretary is Dr. G.J. Faichney, CSIRO Division of Animal Production, P.O. Box 239, Blacktown, N.S.W., 2148. The Society's most recent conference included Symposia on assessment of nutritional status, effect of nutrition on exercise and athletic performance (in humans and animals) and foetal development.

The diversity of ethnic backgrounds, the impact of European and more recent Asian migration, and the influence of convenience foods and sophisticated food processing and marketing practices are influencing the way in which

nutritionists are approaching nutritional studies in Australia. There are particular sections of our population which require special attention; the children of recently arrived migrants are one of these. The Aboriginal minority also have special problems because of the prevalence of undernutrition and related infectious diseases in their infants and children. The long-term nutritional consequences of excessive alcohol intakes in Australians and the nutritional needs of an increasingly aging population are also attracting attention.

Australia is a wealthy, industrialized, well-fed nation living on the door-step of South-East Asia and many of the world's poorest and under-fed countries. This geographical uniqueness presents us with national professional challenges which have not yet been adequately met. Some individuals and institutions are now showing the way and it is to be hoped that nutritionists will see this as an important area for mutually beneficial international collaboration in this Region for the future.

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INDIA

Nutritional Deficiencies

Frank nutritional deficiency diseases are widespread in poor communities, particularly among children and pregnant women. Protein-energy malnutrition (PEM), vitamin A deficiency and anemia are the three most common types of deficiency disorders. Research efforts continue to investigate these problems and to evolve methods for their control. Highlights of recent studies in these areas are presented here.

Protein-energy Malnutrition. An important concept that has emerged in recent years is that there is no primary protein deficiency among populations subsisting on predominantly cereal based diets and that the main bottleneck in the diets of even preschool children is calorie inadequacy. As a result, the earlier theory of two separate dietary etiologies for kwashiorkor and marasmus has been challenged. Recent studies on hormone levels have helped in understanding the probable mechanisms of evolution of these two syndromes. Plasma levels of cortisol were found to be raised in both kwashiorkor and marasmus but to a greater extent in marasmus. The response of adrenal cortex to an injection of corticotropin was exaggerated in marasmus but not in kwashiorkor. Plasma growth hormone levels and their response to stimuli were, in contrast, raised in kwashiorkor but not in marasmus. Low levels of somatomedin may be acting as a feed back stimulus resulting in the high levels of growth hormone in kwashiorkor (1). Based on these observations it has been postulated that a good adreno-cortical response is necessary for adequate adaptation leading to marasmus, and that breakdown of this adaptation process leads to kwashiorkor.

Nutritional Blindness. Keratomalacia is often associated with protein energy malnutrition. There is experimental evidence to show that protein deficiency can influence retinol binding protein and vitamin A metabolism. In view of these observations it has been suggested that protein deficiency may play an important role in the development of nutritional blindness in children. Recent studies have shown that in malnourished children with corneal lesions, although serum vitamin A concentration was considerably lowered, the concentration of

retinol binding protein did not fall to the same extent (2). Administration of vitamin A to such children not only produced an increase in serum concentration of vitamin A but also resulted in rapid clinical improvement. These results indicate that vitamin A, rather than the carrier protein, is the main limiting factor.

One of the intervention strategies suggested for prevention of xerophthalmia is periodic administration of massive dose of vitamin A to children at risk. Such a program has been in operation in India for the last 10 years. Although it has been demonstrated that the massive dose is effective in reducing the incidence of mild conjunctival signs of vitamin A deficiency there is as yet no evidence as to its effect on corneal lesions. Recently, a study has been conducted in Hyderbad to assess the impact of the massive dose programme on the inflow of keratomalacia cases into the hospitals. The results of the study showed that there was a significant reduction in the incidence after the programme was started. None of the children who received vitamin A developed corneal lesions indicating that administration of the vitamin can offer protection against nutritional blindness (3).

Nutritional Anemia. Iron deficiency anemia is widespread in poor communities inspite of seemingly adequate dietary intake of iron. Poor absorption of iron, due to high amounts of phytate in cereal based diets, is the major cause of this disorder. The incidence of anemia is particularly high in pregnant women and preschool children. Clinical studies have demonstrated the value of regular supplementation of iron and folic acid tablets in preventing anemia in these vulnerable groups. Such a programme is now in operation in several parts of the country. More recently, fortification of common salt with iron as a possible public health approach to control anemia has been investigated. The technological problems associated with fortification have been overcome and a satisfactory mix has been developed. Field trials have shown that introduction of common salt fortified with iron at a level of 1 mg/g salt was associated with a significant reduction in the prevalence of anemia in rural communities (4).

Nutrition and Immunity

Clinical and epidemiological observations have indicated that infections occur frequently and with greater severity in malnourished children. In recent years studies have been undertaken to explore the mechanisms by which malnutrition predisposes to infection. Bactericidal capacity of PMN leukocytes was found to be significantly impaired in children with kwashiorkor. This defect has been attributed to decreased activity of NADPH oxidase with a consequent reduction in the formation of hydrogen peroxide. The number of circulating T lymphocytes as well as their response to mitogens are significantly reduced indicating that cell-mediated immunity is depressed in severe PEM. Serum thymic hormone activity was reduced in infants and young children with PEM (5). However, the humoral response showed variable results. While the antibody response to typhoid vaccine was significantly impaired, the response to tetanus and diphtheria antigens was unaltered even in severely malnourished children. Secretory IgA levels were low in duodenal fluid and other secretions of the body. Serum complement and lysozyme levels were also found to be significantly reduced in severe malnutrition. More recently studies were undertaken to investigate the role of macrophage in the immune response. In vitro macrophage transformation and the bactericidal activity against E coli were unaltered in PEM (6). However, in vivo macrophage function may be influenced by other factors such as chemotactic and migration inhibition factors. Monoclear chemotaxis has been shown to be impaired in children with kwashiorkor (7).

These studies thus indicate that several facets of immunocompetence are affected in severe malnutrition. However, none of the immune functions tested showed alteration in children with mild PEM. The CMI response as well as antibody response to specific antigens were satisfactory in undernourished children. Mantoux conversion following BCG vaccination and elaboration of LMI factor in response to PPD were also unaltered (8,9). Thus the immune status of a great majority of children who suffer from milder grades of malnutrition, as judged by growth retardation, is satisfactory. It is to be expected, therefore, that such mild forms of malnutrition may not modify the morbidity pattern. This is supported by the observation that in areas where supplementary feeding was implemented, there was no significant change in the morbidity rate. Environment appears to be more important than nutrition in determining infection rates in the community. Based upon poor antibody response to bacterial antigens seen in children suffering from kwashiorkor, it has been suggested that national immunisation programmes may not be effective in communities where malnutrition is widespread. However, recent studies indicate that even in poor undernourished communities a great majority of children would respond satisfactorily to active immunization procedures.

Nutrition, Lactation and Fertility

Recent studies have confirmed the traditional belief that lactation prolongs postpartum amenorrhoea and provides some degree of protection against pregnancy. There is a good correlation between mean duration of lactation and interpregnancy interval (10). Data obtained on undernourished Indian women have shown that maternal nutritional status does not influence the duration of lactation, quantity or quality of milk produced (11). Recently, studies were undertaken to evaluate the effect of maternal nutritional status on the return of menstruation and fertility in a group of lactating women belonging to low income groups(12). Prolonged and unsupplemented lactation was common among these women. Mean duration of lactation was significantly shorter in women whose body weight was more than 55kg as compared to those with lower body weight. The duration of lactation amenorrhoea showed a progressive reduction with increasing body weight even when duration of lactation was kept constant. These studies indicate that improvement in nutritional status may lead to faster return to menstruation in lactating women, which in turn may shorten the interpregnancy interval. It is essential, therefore, that the efforts to improve health and nutrition of lactating women should be integrated with adequate contraceptive care.

Nutrition and Drug Metabolism

Drugs are widely used for treating infections and illnesses which co-exist with malnutrition. The pharmacological/toxicological activities of the drugs are determined to a large extent by the capacity of the individual to metabolise them. Recently studies were undertaken with some of the commonly used drugs to determine whether malnutrition modifies their metabolism.

Tetracycline. Absorption of oral doses of tetracycline was significantly reduced in undernourished subjects (13). Irrespective of the route of administration, the plasma half life of the antibiotic was significantly shorter, the elimination rate being 20% in undernourished subjects as against 10% in well-nourished population. However, the total body clearance remained unchanged.

Chloramphenicol. Administration of a single oral dose of chloramphenicol to malnourished children resulted in a delayed but higher plasma peak levels as compared to the normal. They excreted larger amounts of unchanged drug, indicating a slower rate of conjugation.

Sulfadiazine. Half life of the drug was found to be higher with a reduction in protein bound compound, and with a significantly faster metabolic clearance in undernourished subjects (14). However, despite these alterations the steady levels of repeated doses of sulfadiazine at intervals of 8 hrs remain within the therapeutic range.

Antipyrin. The metabolic clearance rate was significantly higher in the undernourished population who were chronically starved and also exposed to environmental factors like smoking, alcoholism and pesticides. On the other hand, in severe nutritional edema, the clearance rate was significantly reduced with wide variation in individuals investigated. Obviously two opposing factors are responsible for these results and the extent of liver involvement will determine the ultimate response.

Phenylbutazone. The elimination half life was significantly shorter and plasma clearance accelerated in undernourished patients (15). Plasma protein binding was only 86% compared to 96% in normal subjects. Significant correlation was observed between serum albumin concentration and protein binding.

Nutrition and Food Toxics

The consumption of toxic contaminants in foods leads to harmful effects in man and animals. In recent years, several human disease outbreaks in India have been attributed to such food toxins.

Mycotoxins. Toxic metabolites produced by fungi, such as aflatoxins and clavine alkaloids, have been shown to be present in many agricultural commodities. A recent investigation into an outbreak of acute hepatitis in tribal populations of Rajasthan and Gujarat has shown that it was due to consumption of maize contaminated with aflatoxin. This is perhaps the first observation directly incriminating aflatoxin in food as a health hazard in the community.

Some of the newer varieties of *bajra* grown in several dry belts of the country are believed to be relatively more susceptible to ergot contamination. Recent investigations have shown that the ergot alkaloids which contaminate *bajra* and the toxic manifestations resulting from their ingestion are different from those due to ingestion of wheat and rye ergot alkaloids. Methods to identify and quantitate these alkaloids have been developed and the safe limit of contamination has been worked out.

Toxic alkaloids. Outbreaks of epidemic dropsy due to consumption of edible oils adulterated with argimone oil have been reported from different parts of India. During an investigation of the outbreak in Sirpur, it was found that all the oil samples collected from the households of affected subjects contained the toxic alkaloid-sanguinarine. This fluorescent compound could be detected in blood and urine of many patients.

Recently there was a disease outbreak characterized by ascites in tribal population of Madhya Pradesh. Clinical and histological pictures resembled veno-occlusive disease attributed to consumption of bush tea in the West Indies. Investigations revealed that the disease was due to consumption of millet contaminated with Crotalaria seeds containing toxic alkaloids.

Toxic amino acids. Many people are crippled by the excessive consumption of the legume, Lathyrus sativus (kesari dal) a problem which is endemic in some parts of Central India. The free aminoacid B-N-Oxalylamine-L-alanine (BOAA) isolated from L. sativus seeds has been shown to produce the neurotoxic effects. Simple methods by which the toxin can be removed have been worked out making this food safe for human consumption.

Fluoride toxicity. Endemic fluorosis with skeletal involvement has been recognised as a public health problem in several parts of the country. A new clinical manifestation of fluoride toxicity, genu valgum, was identified in some parts of Andhra Pradesh. Biochemical studies have shown that subjects with genu valgum had higher rates of total body calcium turnover as compared to those without genu valgum (16). The reasons for the new clinical manifestation are not clear. This condition is seen more frequently in sorghum eaters than in rice eaters. Analysis of sorghum samples grown in this area showed that they contain more molybdenum compared to grains obtained from other areas. Also, the copper content of drinking water in the endemic area was found to be lower than in control areas. Studies in human volunteers have shown that the urinary excretion of copper increased significantly when intake of molybdenum was increased either given as salt or ingested through natural foods. It is generally believed that the single most important factor that determines the development of fluorosis is the total amount of fluoride ingested. Results of the recent studies suggest that alteration of trace element metabolism may be related to the development of genu valgum associated with gluorosis.

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EDITORIAL

Mucosal Immunity and Macromolecular Absorption

The possibility of independent mechanisms of defense operating on the external mucosal surfaces had been first suggested in the mid-late 19th century. However, an immunochemical definition of the components of the mucosal associated lymphoid tissue has become available only during the past two decades, beginning with demonstration of secretory IgA in human colostrum and milk by Hanson, and in many other external body fluids by Tomasi and his colleagues. During the past decade, it has become quite clear that the mechanisms of mucosal immunity are quite complex. Furthermore, increasing evidence suggests that the role of mucosal lymphoid tissue in the overall immunologic homeostasis is more important and of a far greater magnitude than believed previously (1).

The gut associated lymphoid tissue (GALT) and intestinal mucosa represent two important components of the secretory immune system, because of their major role in the seeding of immunocompetent cells to other mucosal sites and in the systemic uptake of a variety of microbial and dietary antigens and other environmental macromolecules. The Peyer's patches and other organized lymphoid follicles in the GALT contain antigen-reactive precursor immunocompetent B and T lymphocytes. About 40% of these cells possess T cell markers and 60% bear L-chains. Lymphocytes in the Peyer's patches, mesenteric lymph nodes and thoracic duct possess alpha-chain determinants and 85% bear IgA on their cell surface (2). Recent studies have demonstrated the presence of class specific T suppressor cells and helper cells in the Peyer's patches. However, the relative proportion of T suppressor activity appears to predominate in the GALT. This impression is based on the observations that Peyer's patches exhibit paucity of, antibody-dependent cell-mediated cytotoxicity, presentation to and processing of antigens by Peyer's patch macrophages, and antibody production in situ to a variety of antigens administered orally or systemically (3,4). In addition, generation of antigen specific T suppressor cells in the Peyer's patches has been demonstrated following oral administration of certain antigens (5). More recent studies have begun to provide an immunologic profile of a population of unique lymphoid cells intimately associated with the mucosal epithelium referred to as the intra-epithelial lymphocytes. These cells possess cytoplasmic granules, mediate polyclonal T cell cytotoxicity and bear markers of T suppressor cells. A large proportion of these cells are morphologically similar to mast cells and are believed to be thymus derived. Although their clinical significance remains to be fully defined (6,7), their relative proportion has been observed to be increased in certain forms of inflammatory bowel disease. It appears that cells in the Peyer's patches and bronchus associated lymphoid tissue (BALT) represent independent sources of specifically sensitized antibody producing cells for the lamina propria of the GALT and BALT respectively, and for other peripheral mucosal sites such as the mammary glands and the products of lactation, ocular tissue and the genital tract.

Considerable information is now available concerning the functional role of secretory antibody in preventing replication of bacterial and viral agents at the mucosal surface (8). Previous studies from Walker's group have provided convincing evidence to suggest that the secretory antibody may participate in

"immune exclusion" of dietary antigens and other macromolecules by limiting their absorption by a variety of specific and non-specific means of mucosal defense (9). These observations are supported in part by abudant clinical experience of high levels of serum antibodies and immune complexes to dietary antigens and milk proteins in patients with selective IgA deficiency.

Based on the information summarized above, it appears that the GALT and BALT serve as the "wine tasters" of the luminal environment of antigens and are largely responsible for seeing of antigen specific antibody producing cells in the lamina propria of intestine, respiratory tract and other distant immune sites by a complex process of migration and homing of specifically activated lymphocytes. However, a more important role of GALT and possibly BALT may be to significantly limit the development of systemic immune response to certain orally experienced environmental antigens as evidenced by the ease with which systemic tolerance can be induced by the oral route, despite the concurrent appearance of secretory antibody responses (3).

It is reasonable to assume that the physiologic activity of the secretory immune system is a dynamicprocess which operates in a state of equilibrium between the host's internal milieu and the external environment. Some of the host factors which are known to influence the activity of the secretory immune system include hormones; age; route, nature (replicating vs nonreplicating, soluble vs particulate), degree of prior sensitization and the dose of antigenic exposure; and the nutritional status of the host (1,10,11). A series of elegant studies by Chandra (11) and others (12) have provided evidence to indicate that varying degrees of protein-calorie malnutrition profoundly limits the expression of secretory immune response to replicating antigens and alter the response to other environmental agents. However, little information is available regarding the mechanisms underlying the abnormal secretory immunity in malnutrition.

The investigations of Rothman and colleagues published in this issue of Nutrition Research provide important new insight in this regard. These observations suggest that in addition to the defects in the secretory immune function, malnutrition may alter handling and degradation of the dietary and possibly other environmental antigens. The increased antigen uptake by the damaged gut mucosa and the impaired degradation of absorbed antigens by the liver during undernutrition may have important implications in the expression of specific immune responses in the mucosal as well as systemic immuncompetent sites.

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ANALYSES OF COHORT AND AGING EFFECTS ON ANTHROPOMETRIC MEASUREMENTS OF WOMEN IN A LONGITUDINAL STUDY[1]

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ABSTRACT

Selected body dimensions of 34 adult women over 30 years of age were measured at time intervals between 1948 and 1976. Body weight and height were measured on six occasions; chest diameter (breadth and depth), bi-iliac width, calf girth (right), and hand grip (major and minor) were measured on four occasions. The longitudinal data were analyzed by use of appropriate regression models, with independent variables involving year of birth and age of the subjects at the time of the study. There were no significant year-of-birth (cohort) effects for height, calf girth, chest depth, and the hand grip variables. The estimated height function exhibited a significant second-order decline with age after about 45 years. Calf girth and chest depth attained maximum values at about 60 years and had significant second-order age effects. The hand-grip measurements showed significant linear decline over the range of ages of the women. Body weight, body mass index, chest breadth, and bi-iliac width exhibited significant year-of-birth effects. Although body weight and body mass index depended on year of birth of the subjects, their estimated mean values attained maxima at about 50 years of age and showed significant second-order age effects. Weight, body mass index, chest breadth, and bi-iliac width had the largest maximum values for subjects with year of birth near 1895. The chest breadth and bi-iliac width functions had maximum values at earlier ages with later years of birth.

KEYWORDS: anthropometric measurements, aging women, longitudinal study

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INTRODUCTION

The effect of aging upon basic anthropometric variables often has been investigated by considering data obtained from cross-sectional samples of subjects. Differences in variables among age groups for such individuals have generally been interpreted as changes with age. These age effects, however, may have been confounded with influences of other factors, such as cohort effects. This paper considers the analysis of anthropometric variables for a longitudinal study involving a sample of women in Iowa.

The variables that were evaluated included height, weight, body mass index, grip strengths (major and minor hand), calf girth, bi-iliac width, and chest depth and breadth. The term "body mass index" was suggested by Keys et al. (1) for the weight-height index that is defined by weight (kg)/[height(m)]^2. Our purpose was to investigate whether or not there were significant cohort effects for these anthropometric variables and to estimate effects of aging after accounting for their influence.

SUBJECTS AND METHODS

Description of Sample

Anthropometric data were obtained during one to six assessments of the nutritional status of 34 adult women over 30 years old, studied between 1948 and 1976. The subjects were seemingly healthy, white women selected from an area sample of the City of Ames, Iowa, and nearby rural communities. Initial ages ranged from 32 to 82 years. Twenty-six women were in the group that was studied in 1948, with eight being added during the series. Ten died before the final measurements were taken in 1976.

Thirty-one of the subjects had been married, and 27 had borne at least one child. Most of the women were from families with average income; only a few elderly subjects had limited financial resources. More than half of the group had completed 12 years of education and 1 or more years of college work. Thirty-one women were full-time homemakers, but occasionally, some had part-time employment. Three subjects were employed full-time during one or more of the study periods.

Initially, all the women were in good health except for a relatively high incidence of excess body mass. Body weight in relation to height was evaluated on the basis of the body mass index using a "desirable" range of 19-24 as defined by Garrow (2). Sixteen women had body mass indexes within the desirable range; 10 women were in the range of 25-29; the remaining 8 women had values of 30 and over. Thirty-three of the 34 women were measured two or more times between 1948 and 1976. Twenty-five of the 34 women remained in the same initial classification of body mass index; 14 of these 25 women continued to be overweight as indicated by body mass indexes of 25 or more. Throughout the study, the women continued to maintain their own homes and most remained active citizens in the community. Dietary patterns showed little change in intake of food energy with increasing age (3).

Twelve of 34 subjects died since the beginning of the study; their ages ranged from 47 to 89 years at the time of death. Eight women aged 80 years and over and two women aged 67 and 79 years, respectively, died from cardiovascular diseases. Malignant carcinoma was the cause of the death for the other two subjects.

Procedure

Body dimensions measured during the initial (1948) and subsequent examinations (1950, 1958-60, 1963-66, 1968-69, 1976) included height, weight, chest diameters (breadth and depth), bi-iliac width, and calf girth (right). These measurements were taken in the morning according to techniques described by Hrdlička (4, p. 163) with modifications introduced by Ohlson et al. (5). The hand grips (major and minor) were measured with a dynamometer (KYN Scheerer Corp., Germany).

Statistical Analysis

Values of anthropometric variables for a sample subject are postulated to change over time according to the specifications of a regression model involving a quadratic function of the subject's age.[4] If the number of observations on a subject exceed three, then the coefficients of this quadratic aging model and their standard errors can be estimated, given that suitable assumptions are made for the random errors involved. To enable observations on all subjects to be used in the estimation of aging effects, we assume that the coefficients of the aging models for subjects in a given cohort group are the same for all such subjects. In particular, it is assumed that these coefficients are a quadratic function of the year of birth of the subjects. Given these assumptions, it follows that the statistical model for the anthropometric variables is a multiple regression model with nine independent variables involving powers and products of age and year of birth up to the second order.

A further important feature of the statistical model is that the observations on given subjects are assumed to be positively correlated and that the correlation coefficients are the same for all subjects. The random error associated with an observation on a given subject is assumed to be the sum of a "subject effect" and a "measurement-within-subject effect." Given that this "nested-error model" applies, then the coefficients of the multiple regression model involved are not most efficiently estimated by ordinary least-squares methods. Furthermore, tests of hypotheses on the coefficients of the model are not correctly obtained by the standard methods using ordinary least-squares regression. We obtain the generalized least-squares estimates of the coefficients by the method suggested by Fuller and Battese (6) which is available as an option in the computer program, SUPER CARP [see (7)]. Tests of hypotheses on the absence of year-of-birth effects, the absence of age-by-year-of-birth interactions, and the nature of "pure-age effects" are performed by use of the SUPER CARP software.

RESULTS

The basic empirical results for the analyses of the nine anthropometric variables are presented in Table 1. The third column of the table gives the sample values of the F-statistic for testing the hypothesis that there are no year-of-birth effects. We conclude that year-of-birth effects are not all zero if the value of the test statistic exceeds the 95th percentage point of the appropriate F-distribution. (For the F(6, 120) and F(6, 60) distributions these values are 2.18 and 2.25, respectively). Thus, for the first five

[4] The statistical model is presented in more detail in the Appendix. Other variables, such as income, race, education, and occupation, may influence the values of the variables involved, but no account of these is taken in our analysis.

TABLE 1

Empirical Results for the Models of the Anthropometric Variables

Variable	Number of observations	F-test for cohort effects[a]	Estimated coefficients for variables in models[b]						Variances	
			Constant	X_1	X_1^2	X_2	X_2^2	X_1X_2	σ_v^2	σ_e^2
Height (cm)	107	1.49	162.84 (0.82)			-0.061 (0.012)	-0.00365 (0.00048)		23.0 (5.7)	0.66 (0.11)
Major grip (kg)	85	1.12	27.85 (0.99)			-0.193 (0.055)	-0.0043 (0.0023)		17.8 (5.7)	12.5 (2.5)
Minor grip (kg)	84	0.59	24.47 (0.88)			-0.192 (0.058)	-0.0033 (0.0024)		11.1 (4.6)	16.1 (3.3)
Calf girth (cm)	86	0.56	35.21 (0.73)			0.058 (0.021)	-0.00383 (0.00081)		15.9 (4.1)	1.47 (0.29)
Chest depth (cm)	82	0.84	21.32 (0.42)			0.061 (0.020)	-0.00244 (0.00078)		4.0 (1.1)	1.36 (0.28)
Weight (kg)	129	2.96	72.1 (2.7)	-0.35 (0.17)	-0.034 (0.012)	-0.033 (0.051)	-0.0054 (0.0021)		119 (31)	15.4 (2.3)
Body mass index (kg/m^2)	107	2.27	27.06 (0.95)	-0.115 (0.062)	-0.0115 (0.0043)	0.020 (0.024)	-0.00152 (0.00096)		14.0 (3.8)	2.58 (0.43)
Chest breadth (cm)	86	4.18	29.77 (0.53)	0.081 (0.056)	-0.0152 (0.0033)	0.158 (0.043)	-0.0106 (0.0019)	-0.0155 (0.0041)	3.9 (1.2)	1.76 (0.36)
Bi-iliac width (cm)	86	6.64	33.21 (0.52)	0.122 (0.059)	-0.0183 (0.0034)	0.243 (0.047)	-0.0142 (0.0021)	-0.0242 (0.0045)	3.3 (1.1)	2.13 (0.43)

[a] The statistic for testing for the significance of year-of-birth effects has approximately F-distribution with degrees of freedom 6 and (n minus 9), where n is the total number of observations for the variable involved.

[b] The variable X_1 is (Year of Birth minus 1900) and X_2 is (Age minus 50). Where there are no estimated coefficients for some variables, the true parameters are assumed to be zero. Estimated standard errors, correct to two significant digits, are presented below the corresponding coefficients.

variables of Table 1, height, major grip, minor grip, calf girth, and chest depth, year-of-birth effects are considered to be nonsignificant. For body weight, body mass index, chest breadth, and bi-iliac width, year-of-birth effects are highly significant.

Additional analyses revealed that the interactions between age and year of birth were not significant for weight [$F(4, 120) = 2.27$] and body mass index [$F(4, 98) = 1.45$], but were significant for chest breadth [$F(4, 77) = 4.14$] and bi-iliac width [$F(4, 77) = 6.90$]. However, interactions of higher order than age-by-year-of-birth were not significant for chest breadth [$F(3, 77) = 0.73$] and bi-iliac width [$F(3, 77) = 0.05$].

The generalized least-squares estimates for the coefficients of the appropriate aging models are presented in the next six columns of Table 1.

The last two columns of Table 1 present estimates for the among-subjects variance, σ_v^2, and the measurements-within-subject variance, σ_e^2, together with estimates for their respective standard errors. For all variables, the among-subjects variances are significant and are larger than the measurements-within-subject variances, except for the minor hand grip. Thus, under the assumed model, the estimated correlation coefficients for observations on the same subjects vary from 0.41 for minor hand grip to 0.97 for height [$r=\sigma_v^2/(\sigma_v^2 + \sigma_e^2)$].

According to the estimated height model, the coefficients of the second-order age variable is highly significant. The estimated function is graphed in Figure 1. It attains its maximum value of 163.1 cm at age estimated to be 41.7 years, with a standard error of 2.5 years. A plot of the height data suggests that, for the younger age range, the heights may vary about a constant and then decline at a quadratic rate after some particular age. With methods of nonlinear regression, the estimated age at which decreases occur in the mean height function is 44.8 years, with an estimated standard error of 3.2 years. The mean height up to this age was estimated to be 162.9 cm, with a standard error of 0.84 cm. The estimated function after 44.8 years is

$$\text{Mean Height} = 162.92 - \underset{(0.00063)}{0.00416}\ (\text{Age} - 44.8)^2.$$

This model satisfactorily fits the data, but differs little from the estimated quadratic age function.

The estimates of the quadratic-age model for the hand-grip variables indicate that the coefficients of the second-order age variable are not statistically significant. The grip strengths thus decline linearly with increasing age.

For calf girth and chest depth, the estimated coefficients of the second-order age variable are highly significant. The estimated mean calf girth function has a maximum of 35.4 cm at age estimated to be 57.5 years, with standard error of 2.2 years. The estimated mean chest depth function has a maximum value of 21.7 cm at age estimated to be 62.5 years, with standard error of 3.5 years. The estimated calf girth and chest depth functions are quadratic functions of age similar to the height function in Figure 1.

The estimates for the last four variables in Table 1 indicate that the coefficients of the second-order, year-of-birth variable are highly significant. For any particular year of birth, the estimated longitudinal

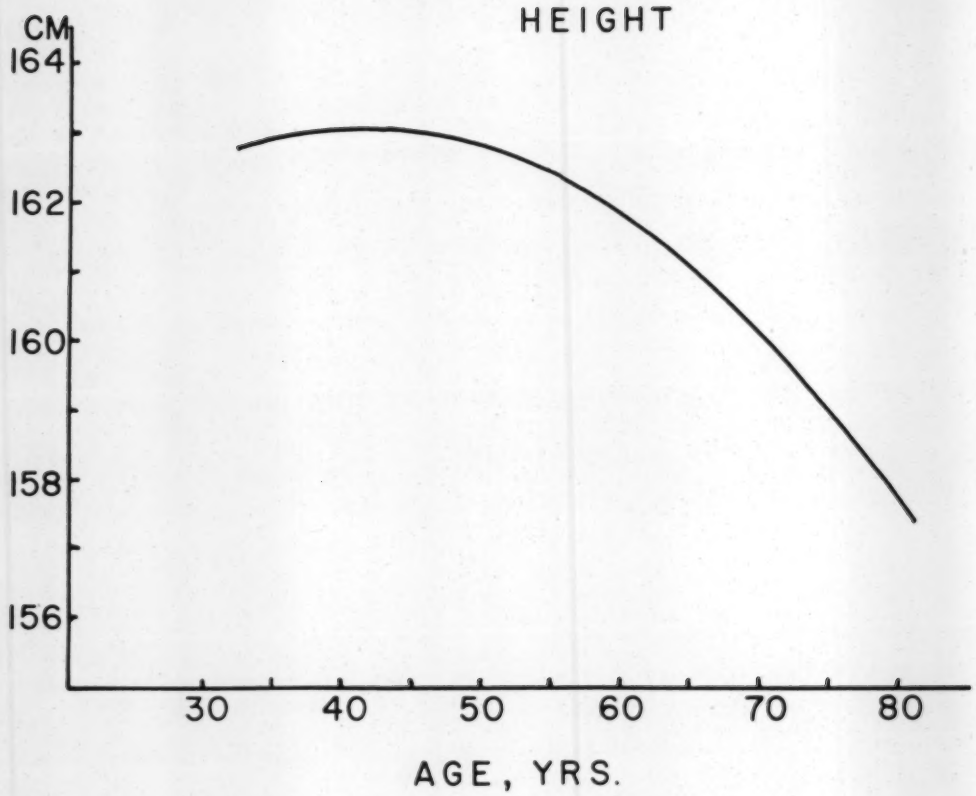

FIG. 1

Estimated mean height of women as a function of their age

weight function attains a maximum value at age 46.9 years (the estimated standard error being 4.9 years). For any given age, the longitudinal weight functions increase until about 1895 and then decline. (The estimated standard error of the estimated maximum year of birth was 3.2 years). The estimated longitudinal weight functions for years of birth 1880, 1885, 1905, 1910 and 1915 are presented in Figure 2. These functions are graphed for a 15 to 20 year age range similar to those for the sample data. It is noted that the weight functions for years of birth equidistant from 1895 are practically identical; that is, the 1905 and 1885 functions almost join, as do the 1910 and 1880 functions.

The mean weights that would be estimated by conducting a cross-sectional study in 1960 are shown by the broken line in Figure 2. The coefficients of this quadratic function are derived from the estimates of the longitudinal weight model (see Section 2 of the Appendix). However, the values on this cross-sectional function are easily deduced from the longitudinal functions. For example, the value of the cross-sectional function for 1960 at age 50 is that for the 1910 year-of-birth function at the same age; the value of the

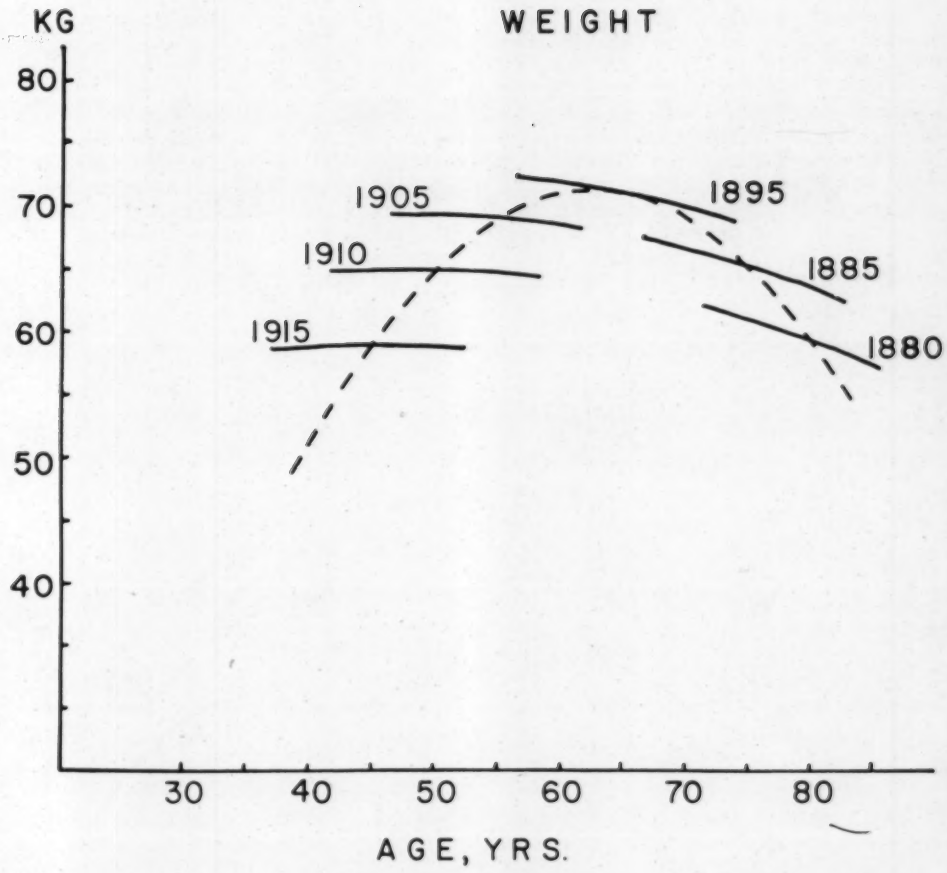

FIG. 2

Estimated mean weight of women as a function of their age for several years of birth. The broken line estimates the mean weights for a cross-sectional study in 1960.

cross-sectional function for 1960 at age 55 years is that for the 1905 year-of-birth function at age 55 years. Cross-sectional weight functions for different study years would be different from that presented for 1960. This graph clearly shows the bias in the estimation of aging effects on weight from cross-sectional studies when cohort effects are significant.

The estimated model for body mass index indicates that there are significant effects associated with year of birth of the subjects and their age at the time of measurement. For any particular year of birth, the body mass index is estimated to have maximum value at age 56.5 years, with a standard error of 8.9 years. The year of birth for which the body mass index has the largest values is estimated to be 1895, with a standard error of 3.3 years.

The estimated means of body mass index for different years of birth are of similar shape to the weight functions in Figure 2.

For the data on chest breadth, the interaction between year of birth and age was significant, and so the year-of-birth functions for chest breadth intersect and have maxima for different ages. These functions for 1880, 1885, 1890, 1900, 1910, and 1915 are presented in Figure 3. The largest maximum chest breadth is estimated to occur at age 58.8 for women born in 1898 (the standard errors being 1.0 and 0.6 years, respectively). The maximum values of the year-of-birth functions occur at earlier ages with later years of birth. The mean values that are estimated by a cross-sectional study in 1960 are shown by the broken line of Figure 3. (The coefficients of this function can be obtained from the estimated longitudinal model by the method illustrated in Section 2 of the Appendix). This function indicates that the biases in the estimation of age effects on chest breadth are likely to be substantial when a cross-sectional study is conducted.

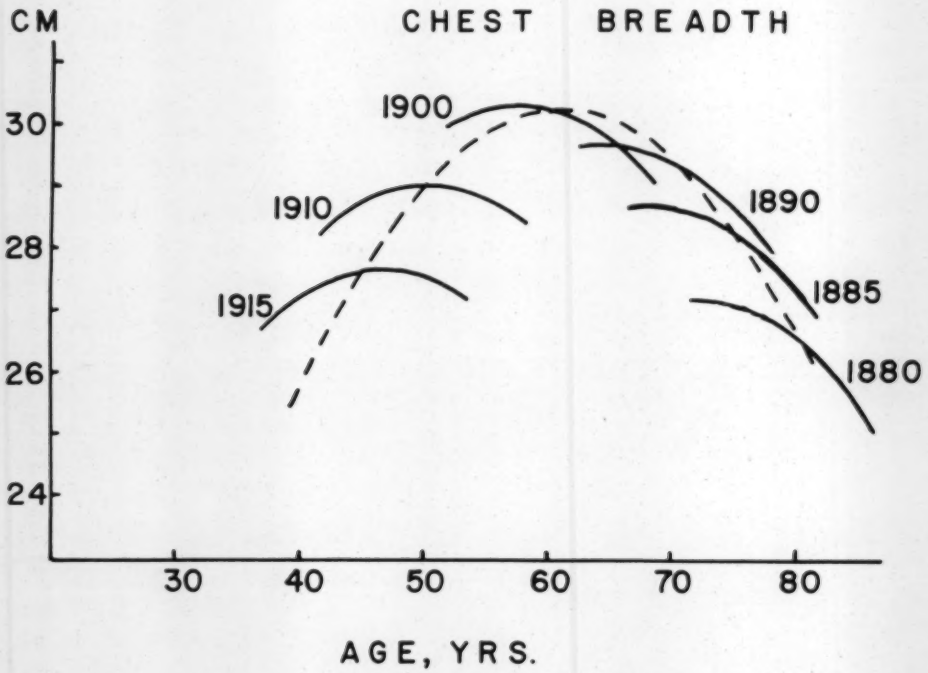

FIG. 3

Estimated mean chest breadth of women as a function of their age. The broken line estimates the mean chest breadths for a cross-sectional study in 1960.

The estimated model for bi-iliac width is of identical form to that for chest breadth. Again, maximum values for the year-of-birth functions occur at earlier ages for women with later years of birth. The largest maximum bi-iliac width is estimated to occur at age 63 years for women born in 1895.

DISCUSSION

In cross-sectional studies, decrements in stature among women in the fifth decade may be interpreted to reflect secular trends. For women over 50 years, the decline in height may reflect both secular trends as well as actual losses in height with increasing age (5). According to Shock (8), the decline in stature observed in repeated measurements on the same men over a 10-year period represents a true loss rather than the results of sampling differences. In our longitudinal study, there were no significant effects of year of birth, but there were significant declines in stature associated with increasing age after about 45 years. These decreases in stature may reflect in part, changes in the vertebrae, with or without a collapse in the vertebral discs. Similar declines in height with age after the fifth decade have been reported in other longitudinal studies (9, 10, 11).

Cross-sectional findings are inconsistent regarding the age of onset of height loss in adult women. Some investigators report no significant change in height with age (12) and others suggest age decrements in stature beginning with the fifth decade (5). According to the 1960-62 Health Examination Survey (13), the average height of adult women remains constant up to age group 35-44 years, then declines progressively. The results of our study are in agreement with this finding. In addition, the lack of significant cohort effects indicates that age trends can be unbiasedly estimated by cross-sectional studies. A recent report on longitudinal observations of healthy white male veterans indicated that height starts to decrease in the fifth decade (14).

Cross-sectional observations of adult white women show a gradual decline in hand-grip strengths from age 30 to 60 years, followed by marked reductions in the later decades (5). Our longitudinal data indicate that the declines in grip strengths with increasing age were essentially constant. The decline in grip strength may be related to alterations in patterns of activity with age or changes in health status of aging individuals or both.

Cross-sectional studies of adult women indicate that body weight increments begin in the fourth decade, with marked gains in the sixth and seventh decades and then a decline thereafter (5, 15). In the 1960-62 Health Examination Survey (13) of the U.S. civilian noninstitutionalized population aged 18-79 years, a maximum average weight of 69.1 kg was recorded for women in the 55-64 age group. Longitudinal observations of California women 50 years of age or more have shown the following average losses in body weights over a 14-year period (1962 data compared with 1948 data): 5.9 kg for 50-59 years, 9.1 kg for 60-69 years, 2.7 kg for 70-79 years (16).

From our longitudinal data, the maximum average weight for women born in 1900 was estimated to be 72.1 kg at age 46.9 years. After 50, the estimated average losses in body weights for successive decades were 0.9, 1.9, and 3.0 kg. The predicted rate of decline from 50 to 70 years thus is smaller than that observed among the California women.

Body weight trends reflect changes in body composition with age. Densitometric measurements of women indicate a rise in body fat from 23.1% in the fifth decade to 55.3% in the seventh decade (17). For groups of women between 18 and 67 years with similar mean relative weights, the mean body density decreased from 1.040 (23% fat) to 1.016 (32% fat) as age increased (18). Determinations with K^{40} have shown that lean body mass remains fairly constant up to about 50 years, with a significant decline thereafter (19). In addition to the influence of body composition changes, the body weight patterns observed in our study may reflect, in part, weight consciousness

among many of the subjects. Women who maintained average weights tended to exert conscious efforts to stay within their preferred weight ranges; a majority of these women were in the fourth and fifth decades. The overweight women, on the other hand, shared a desire to lose weight, but 78% failed to achieve their weight goals.

Longitudinal observations of veteran white males (14) indicated that chest depth increased with age but that chest breadth began to decline significantly in the fifth decade. Bi-iliac width increased progressively as the men aged. The measurements were taken initially and after 5 years. Our data on women showed a significant second-order age effect for chest depth (but no year-of-birth effects) with a maximum value at age 62.5 years. For chest breadth, there was a significant interaction between age and year-of-birth effects, and so estimated maximum values differed for individuals born in different years.

The analyses of the longitudinal data considered in this paper indicated that year-of-birth effects were not significant for certain variables of interest in the study of aging women. Valid estimates of the effects of aging per se on these variables may, therefore, be obtained from cross-sectional observations; estimates of the effects of age on variables for which there are significant cohort effects, however, may be biased.

Similar trends were observed for the dietary intakes of the same group of women (3). Estimated year-of-birth effects were significant for protein, calcium, phosphorus, riboflavin and preformed niacin, but not for energy or the other nutrients examined. Lack of consideration of cohort effects may yield a biased estimate of age effects on intakes of some nutrients. Longitudinal studies are costly in time, money, and personnel. Their justification lies in the light that may be shed on the validity of the interpretation of data from cross-sectional studies of population groups.

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APPENDIX

1. Statistical Model

The statistical model for an anthropometric variable for the subjects is defined by

$$Y_{ij} = \beta_{0i} + \beta_{1i}X_{2ij} + \beta_{2i}X_{2ij}^2 + u_{ij} , \quad i = 1,2,\ldots, t ; \quad j = 1,2,\ldots, n_i , \tag{1}$$

where Y_{ij} denotes the measurement for the i-th subject on the j-th occasion; X_{2ij} denotes the age of the i-th subject when the j-th measurement is taken (coded by subtracting 50); β_{0i}, β_{1i}, and β_{2i} denote unknown coefficients of the quadratic model for the i-th subject; u_{ij} denotes the random error associated with the j-th measurement on the i-th subject; n_i denotes the number of observations on the given variable for the i-th subject; and t denotes the number of sample subjects involved.

The random errors associated with the observations on the sample subjects are assumed to be defined by

$$u_{ij} = v_i + e_{ij} , \quad i = 1,2,\ldots, t ; \quad j = 1,2,\ldots, n_i , \tag{2}$$

where v_i is a component of variation for the i-th subject and e_{ij} is a component of variation for the j-th measurement on the i-th subject. The v_i's and the e_{ij}'s are assumed to be independently distributed as normal random variables with zero means and variances, σ_v^2 and σ_e^2, respectively. It follows that the observations on the same subject are positively correlated [the correlation coefficient being $r = \hat{\sigma}_v^2/(\hat{\sigma}_v^2 + \hat{\sigma}_e^2)$], but that observations on different subjects are uncorrelated.

The variance, σ_v^2, describes the among-subjects variability of observations, whereas the variance, σ_e^2, describes the variability associated with measurement error and effects of variables not included in the model.

As presented in equation (1), the parameters of the aging model may be different for different individuals. A reduced number of parameters is obtained by assuming that the same model applies for subjects in the same cohort. We further assume that the β-parameters are related to year of birth of the subjects as follows:

$$\left.\begin{aligned} \beta_{0i} &= \beta_0 + \gamma_0 X_{1i} + \delta_0 X_{1i}^2 \\ \beta_{1i} &= \beta_1 + \gamma_1 X_{1i} + \delta_1 X_{1i}^2 \\ \beta_{2i} &= \beta_2 + \gamma_2 X_{1i} + \delta_2 X_{1i}^2 \end{aligned}\right\} , \tag{3}$$

where X_{1i} denotes the year-of-birth of the i-th subject (coded by subtracting 1900)[5].

Given the above assumptions, the model that is considered for analysis of the variables for the longitudinal study is a regression model involving independent variables that are products and powers of year of birth and age (X_{1i} and X_{2ij}) up to the second order. For this model describing cohort and aging effects, several hypotheses might be of interest. Two of these are

(a) There are no year-of-birth effects, i.e.,

$$\gamma_0 = \gamma_1 = \gamma_2 = \delta_0 = \delta_1 = \delta_2 = 0 .$$

(b) There are no interactions between age and year of birth, i.e.,

$$\gamma_1 = \gamma_2 = \delta_1 = \delta_2 = 0 .$$

The first hypothesis (a) implies that the coefficients of the original model (1) are the same for all sample subjects. If this is true, then aging effects are correctly estimated by a cross-sectional study. The second hypothesis (b) implies that cohort effects influence the level of the values of the anthropometric variable, but not the change in the values with increasing age. However, if this is true (but cohort effects are present), then a cross-sectional study does not permit changes associated with aging to be correctly estimated. If cohort effects are present, then the parameters of the model that describe pure aging effects are confounded with those associated with cohort effects whether or not interactions between age and year of birth are present. This is illustrated in the next section.

2. Cross-sectional Estimation When Cohort Effects are Present

Suppose that the true model for an anthropometric variable is defined by (1) - (3) but that there are no interactions between age and year of birth of the subjects. The model is thus given by

$$Y_{ij} = (\beta_0 + \gamma_0 X_{1i} + \delta_0 X_{1i}^2) + \beta_1 X_{2ij} + \beta_2 X_{2ij}^2 + u_{ij} , \qquad (4)$$

where the variables are as defined in the preceding section.

If a cross-sectional study is conducted in a given year and one observation is obtained for each sample subject, then the preceding model (4) cannot be estimated because year of birth and age of the subjects in the study year are linearly related. Further, a regression of the Y-observations on the age and age-squared variables does not yield unbiased estimates for the pure age parameters, β_1 and β_2. This is demonstrated as follows:

For a cross-sectional study, it follows that,

Study Year = (Year of Birth) + (Age in Study Year),

and so in the notation of the above variables

---

[5] A more general class of models involves introducing random errors in (3). Such "random-coefficients models" result in more complicated error structures that make efficient estimation very difficult.

$$\text{Study Year} = (X_{1i} + 1900) + (X_{2i} + 50).$$

Thus, if (Study Year minus 1950) is represented by t,

$$X_{1i} + X_{2i} = t \tag{5}$$

is true.

By substituting $X_{1i} = t - X_{2i}$ in equation (4), the model is expressed by

$$Y_i = \beta_0 + \gamma_0(t-X_{2i}) + \delta_0(t-X_{2i})^2 + \beta_1 X_{2i} + \beta_2 X_{2i}^2 + u_i .$$

This reduces to

$$Y_i = (\beta_0 + t\gamma_0 + t^2\delta_0) + (\beta_1-\gamma_0-2t\delta_0)X_{2i} + (\beta_2+\delta_0)X_{2i}^2 + u_i . \tag{6}$$

Thus, the regression of the Y-observations on the age and age-squared values yields estimates for the coefficients in model (6). That is, the estimate for the coefficients of age squared is biased for β_2 if there are second-order, year-of-birth effects (i.e., $\delta_0 \neq 0$) and, furthermore, the estimate for the coefficient of age is biased for β_1.

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DIETARY CALCIUM AND PHOSPHORUS INTAKES OF A SAMPLE OF CANADIAN PRE-MENOPAUSAL WOMEN CONSUMING SELF-SELECTED DIETS.

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ABSTRACT

Daily energy, protein, calcium and phosphorus intakes of 100 women aged 30.3 ± 6.1 years (mean ± SD) living in a University community and consuming self-selected diets were assessed by calculation from three-day dietary records and food composition data, and by analysis of one-day food composites. The daily calculated intakes were 1803 ± 386 kcal (7544 ± 1615 kj), 74 ± 18 g protein, 943 ± 342 mg calcium and 1398 ± mg phosphorus with a mean Ca:P ratio of 1:1.5 (range = 1.1 to 1.6). Analyzed intakes were 1406 ± 396 kcal (5883 ± 1657 kj), 694 ± 292 mg calcium and 967 ± 335 mg phosphorus with a mean Ca:P ratio of 1:1.3 (range = 1:2.3 to 1:1). Mean calculated calcium, phosphorus and protein intakes exceeded the Canadian Dietary Standard (CDS) recommendation and only 8% of the subjects received calcium intakes less than two-thirds of the CDS recommendation. In contrast, the mean energy intake was below the CDS recommendation for women of comparable ages and activity patterns, 14% of the subjects obtaining less than two-thirds of this level. Six percent of the subjects had high phosphorus intakes (>2000 mg/day) and 8% had Ca:P ratios wider than 1:2. Milk and milk products were the major food sources of both calcium (66.7%) and phosphorus (37.1%), and breads and cereals (25.4%) followed by meat and meat substitutes (20.7%) were important sources of energy. Highly significant correlations ($P<0.001$) were noted between analyzed intakes of energy, calcium and phosphorus and those calculated from the corresponding record day. Mean calculated intakes and the calcium and phosphorus densities were higher than corresponding mean analyzed intakes (energy: 20.8%, calcium: 28.2%, phosphorus: 37.8%) ($P<0.01$), but Ca:P ratios were very similar - calculated Ca:P ratio = 1:1.5, analyzed Ca:P ratio = 1:1.3. The calcium content and Ca:P ratios of the majority of these diets appeared to pose no risk to accelerated bone loss in later life.

KEY WORDS: Calcium, Phosphorus, Intakes, Pre-menopausal, Women, Diets.

INTRODUCTION

Calcium levels in the diet have received considerable attention in recent years due to the important role of this mineral in ageing osteopenia (1,2). In contrast, very little information is available on phosphorus intakes of population groups, probably because a dietary deficiency is unlikely to occur and intakes of phosphorus are assumed to be adequate if calcium requirements are met.

The recent widespread use of phosphate food additives in a diet already rich in phosphorus from natural sources has led to concern that dietary phosphorus intakes may become excessive (3). In animal studies, excess dietary phosphorus has been associated with increased bone loss and osteoporosis (4,5,6,7). The possibility therefore exists that excess dietary phosphorus may be a contributing factor in aging bone loss in man. Osteoporosis is the major orthopedic disorder in post-menopausal women in North America and present evidence suggests that the main nutrients implicated in the etiology of this disease are calcium, phosphorus and protein (1,8,9). These nutrients appear to exert their greatest effect on bone mass accumulation during early adulthood.

This study assesses the levels of these nutrients in the diets of pre-menopausal women in an effort to establish whether a dietary imbalance of calcium and phosphorus occurs in this high risk group.

MATERIALS AND METHODS

One hundred pre-menopausal Canadian women (mean age 30 $\pm$ 6.1 years) from a university community and consuming self-selected diets, participated in the study. Consent was obtained from the subjects after the nature of the study had been fully explained to them. The study protocol was approved by the Human Ethics Committee of the University of Guelph. Dietary intakes were calculated from three-day food records using food composition data and via chemical analysis of a one-day food composite collected during the three-day record period.

Subjects completed the dietary records during three consecutive weekdays, recording all foods and beverages consumed (including drinking water) in household measures. Subjects also provided recipes for all prepared food items.

Daily intakes of energy, protein, calcium and phosphorus were calculated using food composition tables. Where necessary, phosphorus values for foods were obtained from company product information and from the literature (10,11,12,13,14,15). Foodstuffs were divided into six major food groups to determine the most important sources of energy, protein, calcium and phosphorus.

Over any one 24-hour period during the three record days, each subject also collected a duplicate portion of all foods and beverages consumed (including drinking water) in plastic containers. These food composites were returned to the laboratory and homogenized in a large stainless steel Waring Blendor (model No. CB5). Beverages were combined gradually with the solid food items during the blending process to produce a single homogenous sample.

After measuring the total volume, two (20 ml) aliquots were transferred from each homogenate into pre-weighed glass vials and immediately frozen at -17 C for subsequent freeze-drying. All analyses were performed in duplicate on each food composite. Approximately one gram from each of of the freeze-dried samples (dried to constant weight) was ashed in a muffle furnace at 650 C. Calcium and phosphorus were determined on solutions of the ash by established atomic absorption procedures for calcium (16) using a Varion 4 atomic absorption spectrophotometer (Techtron PTY Ltd.) and a colorimetric method for phosphorus (17). Gross energy was also determined on a further one gram of each of the freeze-dried composites by bomb calorimetry (18).

The accuracy of the analytical methods was studied by analyzing Orchard leaves, Standard Reference Material No. 1571, National Bureau of Standards (NBS). Mean values for three replicates were: 1.97 g% Ca and 0.20 g% P. Certified NBS mean values (g%) for calcium and phosphorus were 2.09 g% and 0.21 g%, respectively. Analyzed intakes were compared with those calculated from the corresponding record day using the paired t-test.

RESULTS

The average daily energy, protein, calcium and phosphorus intakes calculated from the three-day records are presented in Table 1. Frequency distributions of these daily energy and nutrient intakes are shown in figures 1-4.

Energy intakes ranged from 1049 to 2705 kcal with a mean value of 1803 kcal/day (7544 kj) which is 86% of the Canadian Dietary Standard (CDS)(19) recommended intake for women of comparable ages and activity patterns. Fourteen percent of the women had energy intakes less than two thirds of the CDS recommendation, 59% obtained intakes between 67 and 99% and 27% of the women had energy intakes which exceeded the CDS recommended level.

TABLE 1

Calculated Mean Daily Energy, Calcium, Phosphorus, and Protein Intakes of Pre-menopausal Women

	Mean Daily Intakes	
	Mean±SD	Range
Energy (kcal)	1803±387	1049-2705
Protein (g)	74±18	39-125
Calcium (mg)	944±343	310-1997
Phosphorus (mg)	1398±384	649-2594
Calcium Density (mg/1000 kcal)	531±181	178-1347
Phosphorus Density (mg/1000 kcal)	787±188	395-1501
Ca:P ratio	1:1.5	1:1.1-1:3.2

In contrast, the average daily intakes of protein, calcium and phosphorus (Table 1) were 180%, 135% and 200%, respectively, of the CDS recommendations. None of the women had protein or phosphorus intakes less than two-thirds of the CDS recommended levels, and only 1% and 2% respectively obtained protein and phosphorus intakes below these recommendations. Only eight percent of the subjects had calcium intakes less than two-thirds of the CDS recommended level of 700 mg and 2% obtained intakes between 67% and 99% of this recommendation. The calcium:phosphorus ratios (Ca:P) of these subjects ranged from 1:1.1 - 1:3.2 (Table 1) with a mean of 1:1.5, which is comparable to the 1:1.6 ratio of Canadian diets reported in the CDS (20). The mean calcium and phosphorus densities were 531 and 787 mg per 1000 kcals respectively, and daily intakes of calcium and phosphorus were positively correlated with energy intakes (Ca: $r=.41$, $P<0.001$; P: $r=0.54$, $P<0.001$) and protein intakes (Ca: $r=0.65$, $P<0.001$; P: $r=0.79$, $P<0.001$).

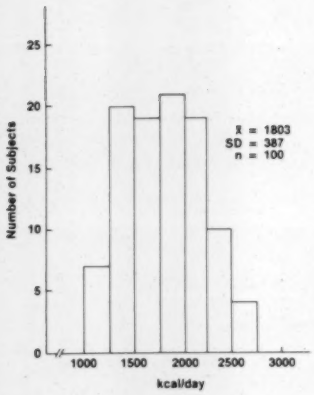

Fig. 1
Calculated mean daily energy intakes.

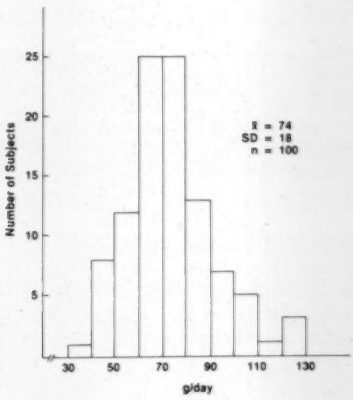

Fig. 2
Calculated mean daily protein intakes.

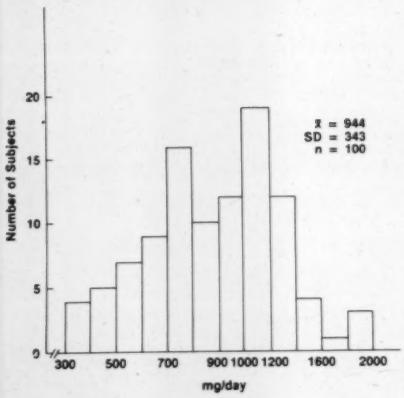

Fig. 3
Calculated mean daily calcium intakes

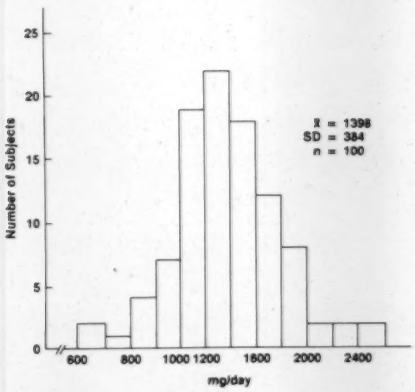

Fig. 4
Calculated mean daily phosphorus intakes

Table 2 presents the percentage contribution of six food groups to the average daily energy, calcium and phosphorus intakes. Milk and milk products were the major sources of both calcium (66.7%) and phosphorus (37.1%) in the diets studied. The major sources of energy were the breads and cereals (25.4%) and meat and substitutes (20.7%), the latter also providing an important source of dietary phosphorus (24.9%).

Table 3 compares the daily intakes of energy, calcium and phosphorus derived from laboratory analyses of the one-day food composite with mean values obtained from calulation of the corresponding record day. Highly significant correlations (energy: r=.69, Ca: r=.74, P: r=.69, P<0.001) were noted for the analyzed intakes of energy, calcium and phosphorus and those calculated from the corresponding record day. The calculated intakes of energy, calcium and phosphorus were 20.8%, 28.2% and 37.8% higher respectively, than the analyzed values, and these differences are statistically significant (P< 0.001). Similarly, mean calcium and phosphorus densities of the calculated versus analyzed intakes were significantly higher (P< 0.001). However, calculated and analyzed Ca:P ratios were comparable (analyzed Ca:P ratio= 1:1.3; calculated Ca:P ratio= 1:1.5).

TABLE 2

Percentage Contribution of Six Food Groups to the Total Daily Energy, Calcium and Phosphorus Intakes of Pre-menopausal Women

Food Group	Energy	Calcium (percentage)	Phosphorus
Milk and Milk Products	18.2	66.7	37.1
Meat and Substitutes	20.7	6.2	24.9
Vegetables	7.3	7.2	8.8
Fruit	10.4	4.5	4.8
Bread and Cereals	25.4	11.1	19.8
Fats, Sugars, Beverages,Alcohol	18.0	4.3	4.6

TABLE 3

Mean Intakes of Energy (kcal), Calcium (Mg), and Phosphorus (mg), Determined by Analysis and Calculated from the Corresponding Record Day

	Analyzed Intake		Calculated 1 day Intake	
	Mean±SD	Range	Mean±SD	Range
Energy	1407±397	695-2734	1700±488	763-3056
Calcium	694±293	157-1802	890±365	196-1990
Phosphorus	967±336	367-2282	1333±406	568-2451

DISCUSSION

The average daily calculated energy intake of the women in this study was comparable to that noted by Taber and Cook (21) in their recent study of American omnivores. Other investigators have reported higher mean energy intakes (22,23,20). These differences can be attributed in part to the relatively sedentary lifestyle of this study group. In addition, variations in dietary methodology, age and socioeconomic status influence estimated or actual energy intakes (22,23,20). Furthermore, the subjects in this study appear to have restricted their food intake on the day of the food composite collection. A similar observation has been noted by White (24).

The major food sources of energy in the diets of these subjects (breads and cereals 25.4%; meat and substitutes 20.7%) were comparable to those found in the Nutrition Canada Survey (20). In contrast, meat, poultry and fish (30.3%) followed by grain products (22.9%) were the major energy sources in the recent U.S. nationwide survey based on 24-hour dietary recalls (25).

Recent research has shown that diets containing high concentrations of isolated proteins (e.g. diets that provide approximately 140 g/day) (26,9,27,28) induce calciuria and negative calcium balance even in the presence of nominally adequate dietary calcium. On the basis of these findings, it was suggested that diets high in protein, such as those rich in meat, may have adverse consequences on bone health in later life. However, when protein-rich foods such as meat were fed in place of isolated proteins, the hypercalciuric effect of protein was offset by the hypocalciuric effect of additional phosphorus, and calcium balance was improved (27,29).

Although there is evidence of parathyroid stimulated bone resorption in adult humans, as in adult animals, following a high phosphorus meal (30), human adults appear to be able to maintain calcium balance over a wider range of phosphorus intakes, apparently by means of a compensating increase in bone formation.

In this study only two percent of the subjects consumed diets containing more than 74 g of protein per day and less than 600 mg of calcium , and none consumed very high levels of protein. Nevertheless the mean protein intake of the group as a whole (ranging from 39 to 125 g/d) was in excess of the Canadian Dietary Standard recommendation (19) and only one subject had an intake slightly less than the recommended level.

Findings of the Nutrition Canada survey suggested the existence of a widespread dietary deficiency of calcium. However, on further analysis this problem was found to exist only for the lower income categories (31). Hence it is not surprising that none of the subjects in this study consumed a diet classified as inadequate in calcium according to the Nutrition Canada Interpretive Standard and only 8% had calcium intakes less than two-thirds of the CDS recommendation. The mean calcium density reported here (Table 1) was also higher than that reported in the Nutrition Canada survey (20) and in other studies (22,24,21).

Milk and milk products were the major food sources of calcium in the diets of the women in this study (66.7%), the Nutrition Canada survey (61%) and the recent U.S. nationwide survey (40.6%) (25) although the percentage contribution from this

food group was smaller in the American compared to the Canadian diets. The absorption of calcium from such diets is estimated to be approximately 40% (32), and with the range of dietary calcium levels reported here, it is unlikely that the subjects were at risk to dietary calcium deficiency.

The average daily phosphorus intake was twice the CDS recommendation and none of the subjects obtained less than two thirds of this recommended level. Other recent Canadian and American dietary studies (22,24,23) have also noted dietary phosphorus levels higher than the CDS recommended levels (20) and the US recommended daily allowances (33). However, phosphorus densities in some of these studies were lower than the phosphorus density reported here (787 mg/1000 kcal) and by Srivastava et al. (34) (787mg/1000 kcal) in their study of the diets of Canadian university students. The major food sources of phosphorus (milk and milk products 37.1%; meat and substitutes 24.9%) differed from those reported in the recent U.S. survey (25) (meat, poultry and fish 32.3%; milk and milk products 23.6%). All these foods and the inorganic phosphate salts used in food processing are sources of readily available phosphorus so that it is likely that 60-70% of the phosphorus content of these diets is absorbed (32)

A Ca:P ratio of 1:1 has been associated with optimal utilization of calcium and phosphorus (35) and recommended calcium and phosphorus intakes are equal in order to ensure adequate phosphorus in the diet (19). The mean Ca:P ratio of the diets studied here was 1:1.5, comparable to the ratio of 1:1.6 calculated from Canadian per capita food disappearance data (36). Ca:P ratios of 1:2 to 1:3 have been shown to increase bone loss in several species of adult animals (37). Eight of the subjects in this study had diets with Ca:P ratios wider than 1:2, (ranging from 1:2 to 1:3.2). It is noteworthy that meat and meat substitutes (eggs, dried legumes, nuts and soya products) were the major sources of phosphorus (31.1%) in these diets, followed by breads and cereals (21.7%), milk and milk products providing only 17.3% and 44.6% of total dietary phosphorus and calcium compared to 37.1% and 66.7%, respectively, in the diets of the group as a whole. Hence these differences in the major food sources of phosphorus and calcium may account for the wider Ca:P ratio of these diets. Mean energy, phosphorus and calcium intakes of these eight subjects were lower than those of the group as a whole and only one of these subjects had an average daily calcium intake which met the CDS recommendation. Despite their lower energy intakes, the mean calcium and phosphorus densities of these eight diets (297 mg calcium/1000 kcal; 673 mg phosphorus/1000 kcal) were also lower than for the group as a whole. While the results of animal studies suggest that the diets of these subjects may have been conducive to bone loss, adult humans may be more tolerant of excess phosphorus than adult animals, and it is not known whether the observed Ca:P ratios were representative of their habitual diets.

Only six women in the study had average daily phosphorus intakes in excess of 2000 mg/d, a dietary level noted by Bell et al. (3) to promote bone resorption. Furthermore, the calcium intakes of these subjects were above the group mean, ranging from 1334 to 1997 mg/day (mean= 1673 $\pm$ 296 mg/d). Hence the mean Ca:P ratio for this subgroup was 1:1.4 (ranging from 1:1.1-1:1.9), very similar to that of the entire group (1:1.5). Although high levels of phosphorus in the diet frequently are associated with processed meats containing phosphate food additives, in this subgroup of

women with high phosphorus intakes, meat and substitutes provided 17.1% of the total dietary phosphorus. Instead, milk and milk products were the most important sources of phosphorus in these diets, providing 45.8% of total phosphorus.

The highly significant correlations between analyzed and calculated intakes of energy, calcium and phosphorus ($P<0.001$) reported in this study have been noted by others (38,24). In this study however, the mean intakes and nutrient densities calculated from the corresponding record day were higher than the mean intakes and nutrient densities derived by chemical analyses of the one-day food composite. In contrast, calcium and phosphorus ratios were comparable. The method of direct calorimetry used in this study determines total energy (18) so that it is noteworthy that the analyzed energy intakes were lower than those obtained by calculation. Acheson et al. (38) reported, as expected, lower calculated energy intakes compared to those obtained by direct calorimetry. Other investigators have compared analyzed and calculated calcium and phosphorus intakes. White (24) reported no differences, whereas Walberg and Adams (39) and Manalo and Jones (40) noted that the calcium content of hospital metabolic diets determined by analysis were lower and the phosphorus content higher than the calculated levels. The latter finding was also demonstrated by Greger et al., (41) in their metabolic diets, composited daily for 51 days.

It is unlikely that the discrepancies observed between the analyzed and calculated energy, calcium and phosphorus intakes were associated with inaccuracies in the laboratory analysis as means for calcium and phosphorus obtained for the NBS Orchard leaves compared very closely to the certified NBS values. Furthermore, inadequate homogeneity of the food composite was also not a source of error as calcium and phosphorus analytical values from duplicates of the 24-hour food composites were comparable. It is possible that subjects failed to include certain foods and beverages in their one-day composite. However, the consistently lower values obtained by chemical analysis for energy, calcium, phosphorus and trace elements (unpublished) suggest that differences in portion sizes were probably responsible in part for these discrepancies. In addition, energy and nutrient values of food items included in any comprehensive food composition table represent weighted averages representative of each food item used throughout the country, on a year round basis (42). Consequently it is not surprising that the energy and nutrient intakes derived via calculation were not identical to those obtained by chemical analysis.

It is of interest that the mean energy, calcium and phosphorus intakes calculated for the one-record day set aside for collection of the food composite were significantly lower ($P< 0.01$) than the mean intakes calculated from the other two days, although the calcium and phosphorus densities were comparable. These results suggest that the subjects altered their total food intake on the day of the food composite collection, a finding also noted by White (24). Hence it appears unlikely that increasing the number of days for the food composite collection would have provided data more representative of the subjects' usual eating pattern. Hence the results of this study suggest that energy, calcium and phosphorus intakes calculated from the three-day food records using food composition data are probably more indicative of habitual intake than those obtained from chemical analysis of a one-day food composite.

In general, the diets of the 100 pre-menopausal women in this study were in compliance with recommendations for daily calcium intake and appear to pose no risk of bone loss attributable to excess concentrations of phosphorus or protein. Six percent of the subjects had high phosphorus intakes (>2000 mg/d) and eight percent had wide Ca:P ratios (1:>2) which, if representative of their habitual diets, may be considered undesirable from the standpoint of bone health.

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FOLACIN AND IRON STATUS OF ADOLESCENTS FROM LOW-INCOME RURAL HOUSEHOLDS (1-3)

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ABSTRACT

The folacin and iron status of 179 black and white adolescents from low-income rural households was evaluated. Red blood cell folacin concentrations were less than 140 ng/ml in 45% of the subjects and 56% of the serum folacin levels were less than 6 ng/ml. Twenty-five percent of all females had low (<16%) transferrin saturation levels in contrast to 12% of the males. Transferrin saturation increased as the male adolescents matured and decreased as the females matured ($p < 0.05$). Anemia (Hb <12 g/dl) was identified in 13% of the black adolescents in contrast to 2% of the whites. Mean cell hemoglobin concentrations and mean cell volumes were lower ($p < 0.05$) in the blacks than in the whites. These findings demonstrate that both folacin and iron inadequacies occur in a significant proportion of this low-income rural adolescent population group.

KEY WORDS: Folacin, iron, nutritional status

INTRODUCTION

Adolescents are particularly susceptible to developing nutritional deficiencies due to their rapid rate of growth and increased nutrient requirements. Numerous surveys have shown that there is a high prevalence of low hemoglobin levels in this age group (1,2), and the data suggest that both adolescent boys and girls are at increased risk of developing an iron deficiency (3,4). In contrast to the numerous reports related to the iron status of adolescents, there is limited information regarding the folacin status of this age group.

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A low-income level may increase the risk of malnutrition (1,2,5), and effective nutrition intervention requires identification of specific nutrition problems within population subsets considered to be at risk (6). In the present study, folacin and iron inadequacies were identified in a significant portion of adolescents from low-income households.

METHODS

The study population included 179 black and white adolescents from low-income households located in a rural Florida county. Households were low-income in that individual household incomes were below 125% of the official poverty income threshold levels. Thus, the sample was heavily weighted in favor of the "poor" and "near poor." Sixty-five percent of the group was white and the sexes were evenly divided for both races. The race-sex combinations included 31 black males, 69 white males, and 52 white females. The ages ranged from 11 to 18 years and 96% were between 12 and 16 (Table 1).

TABLE 1

Number and Percentage of Subjects at Each Age

Age (yrs)	Number of Blacks		Number of Whites		Total Number at Each Age	Percentage at Each Age
	Male	Female	Male	Female		
11	1	0	2	3	6	3.4
12	2	1	12	10	25	14.0
13	2	7	24	17	50	27.9
14	7	6	12	9	34	19.0
15	14	9	13	7	43	24.0
16	4	3	6	6	19	10.6
17	1	0	0	0	1	.6
18	0	1	0	0	1	.6

The procedures followed were in accord with the ethical standards of the Committee for the Protection of Human Subjects of the University of Miami School of Medicine and the University of Florida.

The subjects were paid volunteers recruited from two separate schools. During the clinic visit at each school, a medical history was taken, and each subject underwent a physical examination by a physician trained in the detection of physical signs of nutritional deficiencies. During the physical examination each subject was rated as to the level of sexual maturity by the physician and assigned a sex maturity rating (SMR) according to Tanner's classification (7,8). SMR 1 represents prepubertal development; SMR 2 represents the first visible signs of pubertal change; SMR 3 reflects the onset of the growth spurt; SMR 4 is the period of rapid, but lessening development; and SMR 5 signifies adult development. The number of subjects in each race-sex category at each SMR is shown in Table 2.

Fasting blood samples were taken by venipuncture for complete blood counts and the analysis of serum and red blood cell folacin, serum iron and transferrin saturation by previously reported methodology (9).

TABLE 2

Number of Subjects in Each Sexual Maturity Rating Category

Sex and Race	Sexual Maturity Rating 1	2	3	4	5	Total
Males						
Black	1	4	12	9	3	29
White	16	15	25	10	1	67
Females						
Black	1	6	9	8	2	26
White	9	18	11	9	2	49
All Subjects	27	43	57	36	8	171

Analysis of data employed the use of multiple regression to develop models to predict changes in the biochemical indices as a function of age. Analysis of variance for unbalanced data using the General Linear Models (GLM) procedure in the Statistical Analysis System (SAS) (10) was used to test for differences between Sexual Maturity Ratings and interactions with sex and race for each of the biochemical indices. Duncan's Multiple Range Test was used to determine differences when a significant Sexual Maturity Rating resulted.

RESULTS

The folacin, iron, and hematological data are presented in Table 3. Differences between sexes within each race and between races within each sex are indicated. In studies of nutritional anemias, it is considered preferable to characterize the status of populations by providing frequency distributions rather than by relating it to a single arbitrary value (11). Figures 1-5 illustrate the frequency distribution curves for hemoglobin (Hb) concentration, mean cell hemoglobin concentration, mean cell volume, transferrin saturation, and red blood cell folacin concentration.

Anemia (Hb<12 g/dl) was more prevalent in the black adolescents than the whites (13% and 2%, respectively). None of the white males were classified as anemic (Hb<12 g/dl) in contrast to 4% of the white females and anemia was more prevalent in the black females than in the black males (21% and 7%, respectively). All white adolescents had normal (>32%) mean cell hemoglobin concentrations in contrast to low levels in 15% of the black adolescents. There was no evidence of macrocytosis in any of the subjects. Twenty-one percent of all black adolescents had reduced (<78 μm^3) mean cell volumes in contrast to only 2% of all white adolescents. Twenty percent of both black and white adolescents had transferrin saturation levels less than 16%. Twenty-five percent of all female adolescents had low (<16%) transferrin saturation levels compared to 12% of all males.

Folacin status was estimated by red blood cell and serum folacin concentrations using standards suggested by Sauberlich (12). Sixty-three percent of the black adolescents had red blood cell folacin concentrations less than 140 ng/ml compared to 45% of the whites. Serum folacin levels were less than 6 ng/ml in 68% of the black adolescents compared to 56% of the whites. Red blood cell and serum folacin concentration were positively correlated ($p < 0.0001$).

TABLE 3

Biochemical and Hematological Data of Rural Adolescents*

Parameter	Female		Male	
	Black	White	Black	White
Hemoglobin (g/dl)	(24) 12.8 ± 1.13[a]	(45) 13.4 ± 0.87[a]	(29) 13.6 ± 1.19[a]	(66) 14.5 ± 1.11[a]
Hematocrit (%)	(24) 38.8 ± 2.65[a]	(45) 39.6 ± 2.57[b]	(29) 41.3 ± 3.51[a]	(66) 42.3 ± 3.16[b]
Red blood cells x 10^6	(24) 4.56 ± 0.27[a]	(45) 4.5 ± 0.30[b]	(29) 5.09 ± 0.53[a]	(66) 4.9 ± 0.35[b]
White blood cells x 10^3	(24) 5.72 ± 1.62[a]	(45) 6.2 ± 1.86[b]	(29) 5.35 ± 1.70[c]	(66) 5.7 ± 1.70[d]
Mean cell volume (μm^3)	(24) 84.4 ± 5.79[a,c]	(45) 87.3 ± 3.49[a,d]	(29) 80.7 ± 6.95[b,c]	(66) 85.8 ± 3.69[b,d]
Mean cell hemoglobin (pg)	(24) 27.6 ± 2.47[a]	(45) 29.3 ± 1.27[a]	(29) 26.3 ± 2.58[b]	(66) 29.2 ± 1.23[b]
Mean cell hemoglobin concentration (%)	(24) 32.8 ± 1.07[a]	(45) 33.6 ± 0.68[a,c]	(29) 32.7 ± 0.73[b]	(66) 34.1 ± 0.66[b,c]
Serum folacin (ng/ml)	(20) 4.8 ± 2.96[a]	(43) 5.96 ± 3.41[b]	(26) 5.7 ± 3.19[c]	(56) 8.34 ± 7.09[d]
Red blood cell folacin (ng/ml)	(20)135.0 ± 64.0 [a]	(40)166.0 ± 115.0 [b,d]	(26)124.0 ± 44.0 [c]	(55)224.0 ± 128.0 [c,d]
Serum iron (µg/dl)	(22) 86.2 ± 34.1 [a]	(37) 88.9 ± 38.5 [b]	(28) 89.6 ± 34.3 [c]	(61) 97.9 ± 45.5 [d]
Transferrin saturation (%)	(7) 21.2 ± 12.9 [a]	(17) 27.0 ± 10.8 [b]	(12) 25.8 ± 10.2 [c]	(12) 29.6 ± 10.9 [d]

*Values represent means ± S.D. Numbers in parentheses are numbers in group.

[a,b,c,d] Means within the same horizontal row with a common superscript differ significantly ($p < 0.05$). Comparisons were made between sexes within each race and between races within each sex.

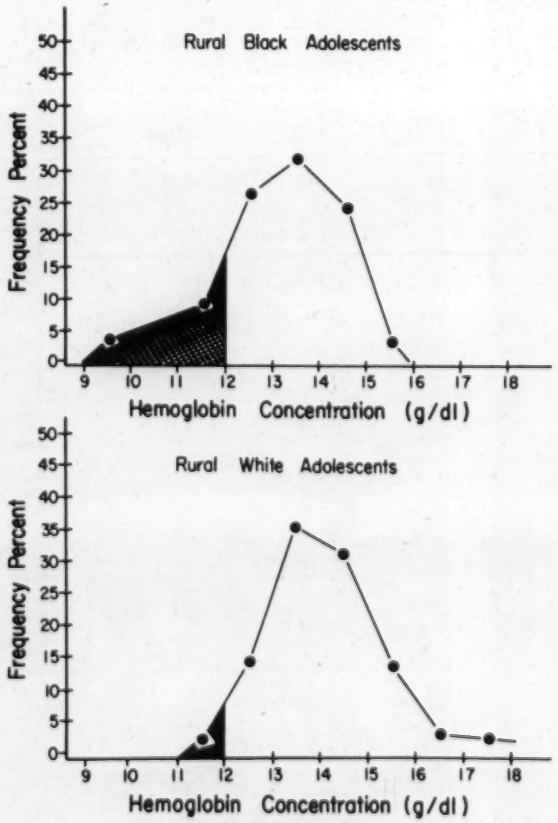

FIG. 1

Distribution of hemoglobin concentrations in black and white adolescents.

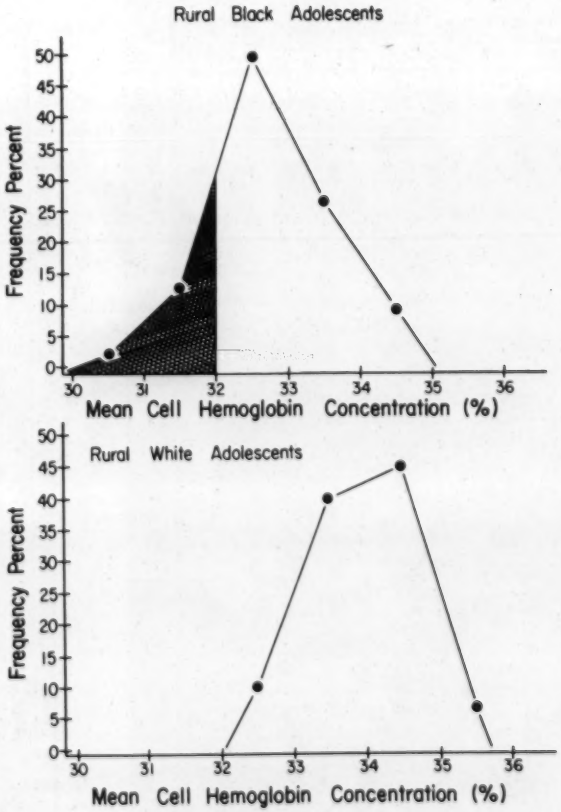

FIG. 2

Distribution of mean cell hemoglobin concentrations in black and white adolescents.

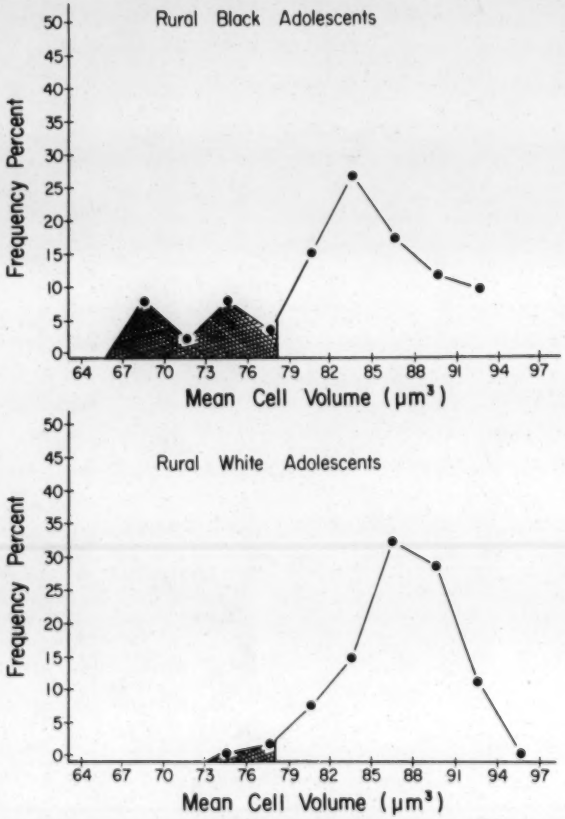

FIG. 3

Distribution of mean cell volumes in black and white adolescents.

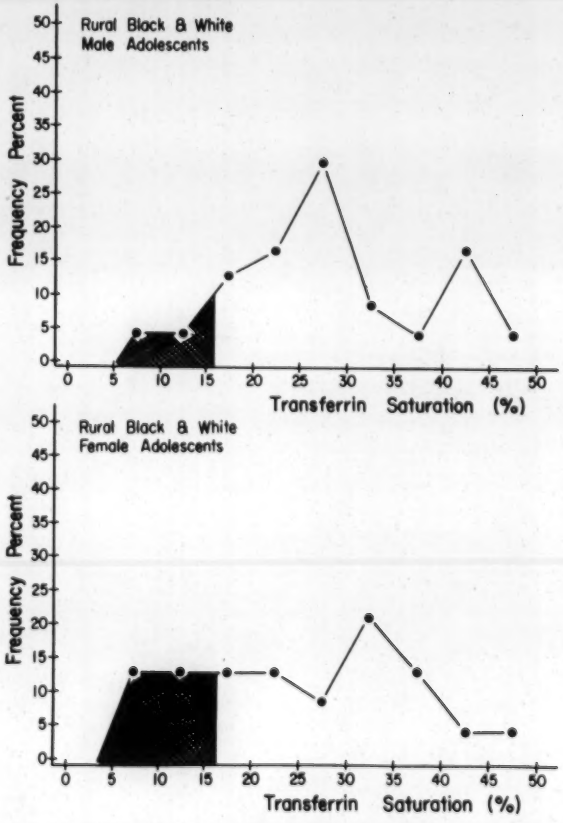

FIG. 4

Distribution of transferrin saturation levels in black and white males and females.

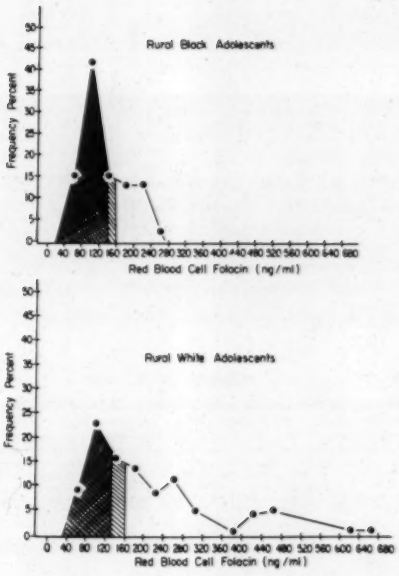

FIG. 5

Distribution of red blood cell folacin concentrations in black and white adolescents.

Serum folacin levels declined over age in male and female adolescents of both races (Figure 6). The resulting regression equation was: Serum folacin = 15.26-0.67 age + 1.81 sex ($p < 0.05$, $R^2 = .05$).

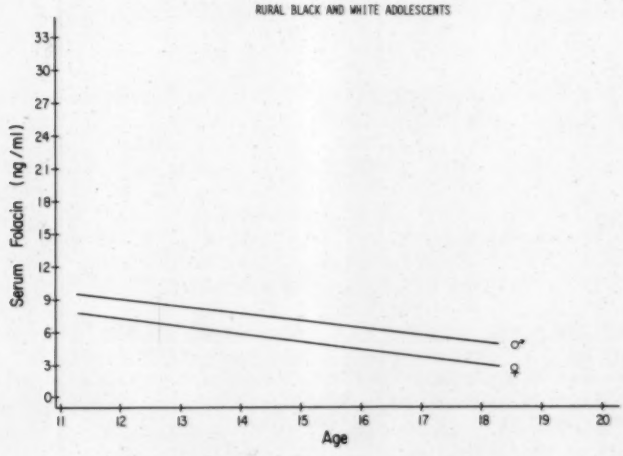

FIG. 6

The relationship of serum folacin to age for males and females.

Adolescents were categorized into specific sexual maturity groups as described by Tanner (7,8) and the mean serum folacin concentration and transferrin saturation levels of each group were compared (Table 4).

TABLE 4

Differences Between Sexual Maturity Ratings (SMR) for Mean Serum Folacin Concentration and Transferrin Saturation as Determined by Duncan's Multiple Range Tests*

SMR	Serum Folacin Concentration (ng/ml) for Males and Females	Transferrin Saturation (%) Males	Transferrin Saturation (%) Females
1	7.7	22	38
2	6.5	18	26
3	7.3	32	21
4	4.8	34	15

*Means connected by same line are not different ($p > 0.05$). Rating 1 represents prepubertal development; Ratings 3 and 4 are associated with the growth spurt. Menarche occurs predominately at SMR 4.

Serum folacin concentrations tended to decrease ($p < 0.10$) as the adolescents matured. Transferrin saturation levels increased in adolescent males as they matured in contrast to a decrease in females ($p < 0.05$).

DISCUSSION

Biochemical and hematological data obtained in the present study indicate that both folacin and iron inadequacies are present in a significant portion of this adolescent population group. These data are important since very little information is available related to the folacin status of adolescents. Conclusions reported in the Ten-State Nutrition Survey (1) have been criticized since they were based on mean folacin levels alone with no indication of the prevalence of values below accepted norms (13,14). While it is generally recognized that red blood cell folacin concentration is the better index of tissue stores of folacin (15), investigators have commonly evaluated folacin status based on serum levels alone (16,17). In the present study both serum and red blood cell folacin concentrations were determined. The finding of such high prevalences of reduced red blood cell folacin concentrations suggests that tissue stores of this nutrient are being depleted. While the functional consequences of reduced tissue stores of folacin are not clearly understood, hematopoiesis would be compromised when stress is superimposed on folacin deficiency. The risk of poor pregnancy outcome is increased in the folacin-deficient adolescent (18). Our research group has recently reported the widespread occurrence of poor folacin status in pregnant low-income adolescents (19).

The finding in the present study that serum folacin levels decreased with increasing age and sexual maturity is in agreement with the report by Daniel et

al. (17). Increased folacin requirement for cell replication during this rapid phase of adolescent growth may account for this decrease.

The hematological parameters indicate that an insufficient supply of iron was present for normal hematopoiesis in a portion of this population. The prevalence of iron deficiency as determined by transferrin saturation levels is in agreement with previous findings (20). Results of the present study suggest that the risk of iron deficiency increases as the adolescent female matures in agreement with the report of Seltzer et al. (21). The decrease in percent transferrin saturation with maturity in the females may be partially explained by the fact that 78% of these girls had begun menstruation. The peak of adolescent growth usually follows the onset of menses and may further increase the demand for iron as the adolescent female matures. In male adolescents, transferrin saturation increased with increasing maturity in agreement with other reports (21,22).

While supplementation with the hematinic nutrient is considered a useful diagnostic tool (4), the importance of surveys to assess the risk of nutrient inadequacies within a population should be recognized (12). More research is needed to determine the functional consequences of marginal iron and folacin status.

The hematological data indicate that anemia and reduction in mean cell volumes were more prevalent in the black adolescents than in the whites. The interpretation of differences in hematological indices between racial groups should take into account possible genetic as well as nutritional factors. A report by Dallman et al. (23) indicates that norms for hemoglobin levels should be lower for black population groups. It has also been reported that α-thalassemia and sickle cell anemia should be recognized as potentialy contributing factors to the prevalence of anemia in Blacks (24, 25). The gene frequency for α-thalassemia in black Americans is estimated to be approximately 25% (26). Thalassemia will result in a reduction in mean cell volume and the hematological profile is one of microcytosis and hypochromia as is iron deficiency (25). The frequency distribution curve for mean cell volume in the Blacks (Figure 3) may reflect a subgroup that has α-/α-thalassemia which is reported to be in the range of 2% for black Americans. The single deletion α-/αα is clinically silent, therefore would not be apparent in these data. Sickle cell anemia was not screened for and may have contributed to the prevalence of anemia in this study.

In summary, these data suggest that folacin tissue stores are being depleted and hematopoiesis is being compromised by an insufficient supply of iron. These findings demonstrate that the potential for both folacin and iron inadequacies during adolescence should be recognized and investigated in this age group.

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ESTIMATING NUTRIENT DEFICIENCIES IN A POPULATION FROM DIETARY RECORDS: THE USE OF PROBABILITY ANALYSES

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ABSTRACT

The objective of this report is to describe a probability approach, and to compare approaches, to evaluating dietary intake data. The nutrient intake data used were calculated from seven-day food records obtained from 83 Ontario teenagers and the adequacy of the estimated nutrient intakes was assessed by three approaches. First, mean nutrient intakes were compared to the recommended daily nutrient intakes (RDNI). Second, the proportion of the population not meeting the RDNI was calculated. Third, the distribution of nutrient intakes below the RDNI was determined, and combined with probability statistics to calculate the number of subjects likely to have intakes below their own individual requirements. This latter approach recognizes that the RDNI overestimates the nutrient requirement of almost all individuals in the population. The first approach was found to underestimate, whereas the second approach overestimated, possible problems. The third analysis appears to predict an incidence of nutrient inadequacy which is more in agreement with biochemical evidence. Therefore, given an adequate estimate of usual daily nutrient intakes of the individual, dietary data may be effectively used to identify nutrient problems in a population.

KEY WORDS: Probability analysis, Nutrient intakes.

INTRODUCTION

It has become customary to evaluate nutrient intake data calculated from dietary records by making direct comparison of the intakes with recommended nutrient intakes as published in appropriate dietary allowances. Unfortunately this simple comparison fails to adequately recognize that the recommended intakes [Recommended Daily Nutrient Intakes (RDNI) in Canada (1), Recommended Dietary Allowances in the United States (2)] are a statement of requirement that is adequate to maintain health in almost all healthy individuals. That is, the recommended intake for each nutrient (excluding energy) takes into account the individual variability of requirement. As a result the recommended intake exceeds the actual requirement of almost all individuals. The recommended intake is a level of intake of a nutrient that represents a low risk of inadequacy to the individual. Failure to achieve the recommended intake does not mean the intake is inadequate to meet the individual's own requirement. Conversely, the lower the individual's observed intake relative to the recommended intake, the greater is the probability (risk) that the individual has not met his requirement (3). A simple comparison of nutrient intakes of individuals in a survey population with the recommended intakes gives no indication of the number of individuals in that population who are likely to have intakes below their own individual requirements.

The objective of this report is to describe a probability approach, and to compare approaches, to evaluating dietary intake data. The nutrient intake data were calculated from seven day dietary records obtained from Ontario teenagers. As reported elsewhere the records were from a larger study of 343 Ontario children and were also used to calculate the intake of each dietary sugar, including sucrose (4), and the relationships between sugar consumption and dental health (5).

METHODS DESIGN

The survey sample has been previously described (4). Briefly, students from the chosen schools were invited to participate in the study during June 1978. Written consent was obtained from a parent or guardian of each child after the purpose of the survey and procedures to be followed had been explained. Complete records for 45 males and 38 females were obtained.

A seven-day food consumption diary was completed for each participant. In order to enhance the possibility of accurate recording of the data each family was monitored by a dietitian (4).

The nutrient data base (6) used to analyse the diet of the subjects was based on Handbook No. 456 of the United States Department of Agriculture (7), but was updated with Canadian food composition data (8) and through direct contact with food industries. The nutrient content of home-prepared foods was calculated from the above sources using recipes obtained from The Joy of Cooking (9) and The Better Homes and Gardens New Cookbook (10).

STATISTICAL ANALYSES

To illustrate the concepts expressed in this paper the mean daily intakes and standard deviation of energy, protein, vitamin C, thiamin, riboflavin, iron and calcium were first calculated and compared to the appropriate RDNI (1). As well, the proportion of children not consuming the RDNI was calculated and expressed as a percentage.

These conventional approaches to presentation of dietary data do not take into account the expected variability of individual requirement along with the variation in intake among individuals. As previously noted an overestimation of the incidence of inadequate intakes would be expected in the latter approach (1-3, 11).

The probability approach described by Beaton (3,11) was extended and applied to the data to obtain approximate estimates of the numbers of individuals expected to have inadequate intakes (intakes below their own requirements). To apply this approach the distribution of requirements, as well as intakes, among individuals must be known or a reasonable assumption about this distribution must be made. Further the possible correlation between intakes and requirements must be considered (3). Reasonable assumptions for most nutrients are that (a) the distribution of requirements is Gaussian in nature, (b) the coefficient of variation is about 15%, and (c) there is a very low order of correlation between intake and requirement as long as confounding variables to which both relate are eliminated. Clearly the last assumption does not hold for energy (3,11) and the first assumption is inappropriate to iron in menstruating women (3,12). It is further assumed that the RDNI has been set at two standard deviations above the mean requirement. This is an explicitly stated approach for only some of the nutrients. In the absence of complete information, this appears to be a reasonable approximation at least for purpose of testing an approach to data interpretation.

At 30% (two standard deviations) above the mean requirement, or at the RDNI, the probability of inadequacy of intake is 0.025, whereas at the mean minus two standard deviations, or at 54% of the RDNI, the probability of inadequacy is 0.975 (3,11, Fig.1).

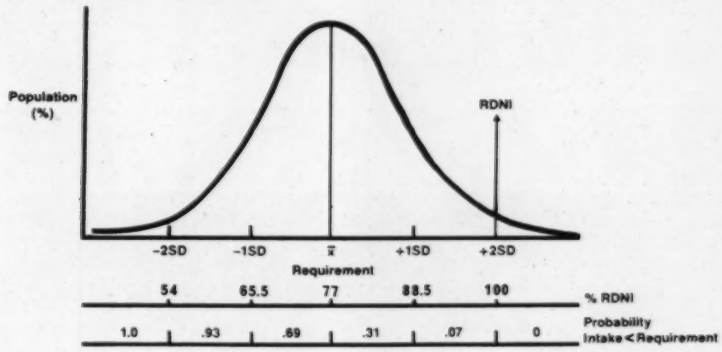

FIG. 1

Description of distribution of nutrient requirements and the probability of intakes being less than the individual's requirement within given intervals below the RDNI.

The probability that intake is less than requirement for the individual at various intervals below the RDNI can be taken from probability tables (13). For the present calculation average probabilities were obtained for intervals of intake conveniently described by a standard deviation of the requirement (Fig. 1). These main interval points on the normal distribution were then translated into a percentage of the RDNI. For example, an intake equivalent to the $\bar{x}$ plus 1 SD would correspond to 88.5% of the RDNI [($\bar{x}$ requirement + 15%/RDNI)x 100]. Thus the intervals chosen correspond to the following percentages of the RDNI; $\geq$ 100, 88.5-99.9, 77-88.4., 65.5-76.9, 54-65.5 and <53.9. The corresponding average probabilities of inadequacy of intake for these intervals are 0, 0.07, 0.31, 0.69, 0.93 and 1.0, respectively.

To obtain an estimate of the proportion of the survey population expected to have an intake below their own requirement for a given nutrient, the following steps were taken. First, the number of individuals with intakes falling within each of the selected intervals was determined. Second, this number was multiplied by the appropriate probability for the interval to give the number of individuals in each interval who were likely to have intakes below their own requirements. Finally the numbers calculated for each interval were summed and expressed as a percentage of the total population.

In the case of iron the above assumptions were applied except for the 16-17 year old females. For menstruating girls it is known that the distribution of requirements is skewed and the probability estimates derived by Beaton (3) for adult females have been used.

RESULTS

The mean daily intakes of several nutrients are shown for each age, and sex group in Table 1, where they are compared with the recommended intake (RDNI) obtained from the Canadian Dietary Standard (1).

TABLE 1

Daily Energy and Nutrient Intakes Compared to the RDNI

	Sex	Intake	RDNI[b]
Energy (MJ)	M	12.4± 4.1[a]	13.4
	F	8.3± 2.9	8.8
Protein (g)	M	112 ± 43	54
	F	75 ± 30	43
Vitamin C (mg)	M	179 ±116	30
	F	131 ± 86	30
Thiamine (mg)	M	2.2± 1.3	1.6
	F	1.3± 0.6	1.1
Riboflavin (mg)	M	3.9± 2.0	2.0
	F	2.2± 1.2	1.3
Iron (mg)	M	19 ± 9	14
	F	11 ± 4	14
Calcium (mg)	M	1499 ±776	1000
	F	1027 ±628	700

a Mean ± S.D.
b RDNI for 16-18 yr age group (1)

The values reported include nutrient consumption in the form of vitamin and/or mineral supplements. As shown in Table 1 average energy intakes approximated the estimated average energy requirement. In the absence of any detailed anthropometric measures it has to be assumed that those with high intakes have high energy expenditures and vice versa. There is good reason to believe that in unconstrained populations, there is a strong correlation between energy intake and requirement. As a result little can be said about possible excesses or deficiencies of energy intake for individuals in the survey population.

Based on average values the intake of all nutrients except the iron intakes of 16-17 year old females exceeded the RDNI (Tables 1 and 2). However, if the proportion of the children not meeting the RDNI is considered, the apparent profile of dietary inadequacy is as shown in Table 2.

TABLE 2

Comparison of Approaches to Dietary Assessment of Nutrient Inadequacy

Nutrient	Sex	Mean Intakes (%) RDNI	Proportion Below RDNI(%)	Probability Estimate of Inadequacy (%)
Protein	M	207	2	2
	F	174	13	2
Vitamin C	M	597	2	0
	F	437	5	3
Thiamine	M	137	31	14
	F	118	44	24
Riboflavin	M	195	9	4
	F	169	24	8
Iron	M	136	24	12
	F	79	84	20
Calcium	M	150	24	11
	F	147	34	21

Substantial proportions of the population failed to consume the RDNI, with the proportions being most apparent for the females.

The results of probability prediction of the proportion of children likely to have intakes below their own requirement are also shown in Table 2. Compared to the relatively large numbers not achieving the RDNI the probability estimate suggests fewer children with intakes below their own requirements. However, the overall trend toward higher proportions for the females than the males is still apparent.

DISCUSSION

Tables 1 and 2 present three different perspectives of the dietary status of the studied teenagers. First, when group mean intakes are expressed as a percentage of recommended intakes (Table 2), the dietary intakes appear to be more than adequate. This bears analogy to the comparison of per capita intake with demographically weighted RDNI's. However, this approach ignores the distribution of intakes among individuals. It clearly underestimates the likelihood of inadequate intakes.

Second, when individual intakes are compared with appropriate RDNI's (Table 2), the distributions of intake is partially considered, allowing one to calculate the proportion with intakes below the RDNI. However, this calculation ignores the distribution of requirement among individuals, and the full distribition of the intake. It clearly overestimates the actual incidence of inadequate intake (intakes below individual requirements).

These two approaches, both of which are found in the literature err in opposite directions. One underestimates nutritional problems; the other overestimates the problems. In each case, the issue relates to proper recognition of the variabilities of intake and of requirement.

When both of these variabilities are taken into account in the probability approach (Fig. 1), the portrayal of the population situation is intermediary and seems likely to be much closer to the real situation. Beaton (3,12) has pointed out that for iron in adult women, the predicted prevalence of inadequate intake is quite consistent with haematologic indices of compromised iron status. Therefore, it will be of interest to compare, in the same population, prediction of incidence of nutrient deficiency using this probability analyses with actual biochemical evidence of nutritional deficiency. Given a correlation and given that a nutrient data base is maintained and that food intake records are sufficient to describe usual intakes of the individual it may be possible to monitor changes in the nutritional health of a population without turning to the greater expense associated with biochemical assessment. Biochemical assessment might then be used more effectively to confirm predicted problems.

In the particular analyses conducted in the present paper it was necessary to make assumptions about the variability of requirement and about the relationship between average requirement and RDNI. The assumptions and approaches adopted by the committee responsible for the 1975 revision of The Dietary Standard for Canada were not explicitly stated. It is to be hoped that future committees, in Canada, in the United States and internationally, will be explicit in this regard so that their recommendations can be properly interpreted.

The present calculations, then, must be seen as a first approximation. Emphasis is given to the underlying principles rather than the particular estimates.

Attention must be directed to one other facet of the interpretation of dietary data. There must be a reasonable description of the distribution of usual intakes. Beaton et al (14) have pointed out that with one day records the observed variance is much greater than that of usual intakes. Further the magnitude of the day-to-day variation within individuals which causes the inflation of apparent variance, differs among nutrients. For many nutrients 3-7 days of observations would provide a reasonable portrayal of the distribution of usual intakes. Although for some nutrients with very large day-to-day variations, such as vitamin A, many more days of observation would be needed before reliable estimates of the distributions could be obtained. If the variation of intakes is overestimated (too small a number of replicate observations or too short an observation period) even the probability approach will overestimate the incidence of inadequate intakes. This limitation is not overcome by increasing group size. Ignoring this aspect has been a major problem in many reported surveys.

In summary, the data presented emphasize that there are appropriate approaches to interpretation of nutrient intake data obtained on populations. Probability analyses may prove to be a useful approach to evaluating adequacy of nutrient intakes calculated from dietary records. Because this analysis takes into account both the distribution of nutrient requirements and of nutrient intakes the proportion of individuals predicted at risk of nutrient deficiency becomes much lower than if the proportion of individuals failing to meet the RDNI is calculated. Furthermore, the incidence of deficiency predicted by probability analyses may prove to be correlated with incidence of biochemical deficiency in groups. If so, dietary data could be used effectively to predict nutrient problems in a population.

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ANALYSIS OF SELF-REPORTED EATING AND RELATED BEHAVIORS IN AN ADOLESCENT POPULATION

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ABSTRACT

A seventy-eight item questionnaire was constructed to collect data from adolescents about behaviors and attitudes regarding eating, food, weight, and physical appearance. It was administered to 2276 students between the ages of 13 and 18. One objective of the research was to identify clusters of behavioral and attitudinal characteristics which may be indicative of disturbed eating and other food-related behaviors. Another was to describe a large sample of adolescents in terms of such characteristics as weight for height and age, perceived ideal weight, and perceived physical attractiveness. Frequencies are reported, as well as the results of a factor analysis. A principal components factor analysis using varimax rotation defined eight orthogonal factors with eigenvalues greater than one. A number of these factors have implications for the increasing incidence among adolescents of such serious eating disorders as bulimia and anorexia nervosa.
Key words: Adolescence; Eating Behavior; Food Habits; Appetite Disorders; Anorexia; Bulimia

INTRODUCTION

Very little information exists on the epidemiology of eating and food-related behaviors and attitudes that may be precursors of more serious eating disorders such as anorexia nervosa (self-starvation), bulimia (binge-eating), and obesity. Each of these serious disorders is manifested by a conspicuous disturbance in eating behavior. Anorexia nervosa and bulimia are exhibited almost exclusively by females, and usually begin in adolescence(1). Obesity affects both sexes; in childhood and after age 50, females in Western cultures have higher obesity rates than males(2).

The research problem was to identify and assess the prevalence of clusters of behavioral and attitudinal characteristics which may be indicative of disturbed eating and food-related behaviors in a group of male and female adolescents. The problem was also one of description: to describe a large sample of adolescents in terms of such characteristics as weight-for-height-and-age; perceived ideal weight; preoccupation with weight and thinness; incorporation of physical exercise, limitation of calories, self-induced vomiting or laxatives as routine aspects of eating and

food-related behavior; and various social correlates of disturbed eating behaviors. A major goal of this research was to provide an epidemiological background against which clinical cases of disordered eating behavior in adolescents having other than an exclusively physiological etiology can be assessed.

A comparison of The Diagnostic and Statistical Manual of Mental Disorders editions II(3) and III(4) indicates that there has been an increasing refinement of the descriptive categories for psychopathological eating disorders. In DSM II, eating disorders were not defined as a diagnostic category; rather, the subcategory Feeding Disturbances was located under the category Special Symptoms. Anorexia nervosa was mentioned as an example of a condition that could appropriately be subsumed under the category Feeding Disturbances. Bulimia was not mentioned. In DSM III, however, Eating Disorders is defined as a category with five subcategories: anorexia nervosa, bulimia, atypical eating behavior, rumination, and pica. (The latter are two rare disorders that characteristically begin in infancy or early childhood.) Obesity is considered to be a physical disorder except in those specific cases where psychological factors are of importance in the etiology or course of the condition.

In addition to anorexia nervosa, bulimia, obesity, and overweight as major types of eating disorders, there may be atypical eating behavior that combines elements of any or all of these. For example, the range of weight may be within normal limits for height, age, and sex, but it may be maintained only by periodic prolonged periods of abstinence or severely limited intake followed by resumption of a high caloric diet in the absence of binging. Or, purgatives may be used, in absence of anorexia, bulimia, or obesity, to maintain a desired weight.

This increasing speciation of psychopathological eating disorders is perhaps a reflection of what appears to be an increase in the incidence of such conditions(5,6,7,8). This, in turn, may reflect the situation existing in American society wherein we live surrounded by a surfeit of food, with a cultural ideal for physical shape, particularly for females, that emphasizes extreme slenderness. It is not surprising then that the incidences of anorexia nervosa, bulimia, and obesity are higher in females than in males.

There are a number of relevant general population studies of adolescent weight, shape, and related attitudes and behaviors. The Purdue Opinion Panel administered a questionnaire to a stratified sample of American high school adolescents in the mid-1950's, and the responses to questions dealing with weight control showed marked regional variation(9). In the Midwest, 54 percent of the adolescents who responded wanted to gain or lose weight, while 52 percent of Eastern, 50 percent of Southern, and 44 percent of Mountain-Pacific adolescents expressed this desire. Questions about modifying one's shape, or "improving one's figure" showed a similar pattern of responses: 25 percent of Southern, Midwestern and Eastern, but only 13 percent of Mountain-Pacific adolescents indicated that this was a concern.

Hueneman, et al(10) studied approximately 1000 teenagers attending public shools in the state of California over a four-year period. The investigation combined an anthropometric study with a carefully constructed questionnaire regarding teenager's preferences, views and knowledge about food and eating, body configuration, and activity. Fewer females than males admitted to ever feeling hungry. The females indicated that they ate less than the males, and about 50 percent classified themselves as obese whereas only 25 percent were identified as obese in anthropometric terms. In

contrast, the male population did not overestimate its fatness in comparison to such measures. The males' concern at all ages was to gain weight, size, and strength. The females' main priority was to lose weight and to attempt to achieve this through dieting.

An epidemiological study of Swedish teenagers brings us closer to the eating disorder anorexia nervosa. Nylander(11) studied a group of 2370 adolescent females and males ages 14 through 19. Investigation was conducted primarily by means of questionnaires administered under supervision. Most of the females in the study indicated that at some time they felt fat. A minority of the males reported similar feelings. Of the females who thought they were fat, many had attempted to diet. Males seldom reported dieting. A total of 119 cases (12 males, 107 females) reported that they had developed at least three of the following symptoms in connection with loss of weight: anxiety, depression, chilliness, constipation, amenorrhea, mental sluggishness, and diminished performance in school. Fifteen of these females reported that they had lost 10 kg. or more in weight; of these, eight were known to the child psychiatric clinic as cases of anorexia nervosa. Nylander concluded that, if mild cases are included, approximately one in ten females in his study reported experiencing an anorexia-nervosa-like syndrome. He identifies the feeling of being fat as a predeterminant.

Attitudes toward weight and appearance in an adolescent population of 446 females and 145 males were investigated and described by Dwyer, Feldman, Seltzer, and Mayer((12). They found that among females, 43 percent could be categorized as obese or of above average fatness on the basis of triceps measurements, but fully 80 percent wanted to weigh less than they actually did. Males also expressed a desire for body changes, but only those who were actually obese indicated that they wanted to weigh less. Rather, adolescent male preferences were for increases in height, weight, shoulders, and upper arm and chest-cage girth. Both males and females tended to express a desire for smaller size in the areas of the waist, hips and buttocks. The most powerful conclusion to be drawn from this research is that high levels of dissatisfaction with the body were reported by almost all adolescent males and females in the sample.

Dwyer, Feldman, and Mayer(13) looked more closely at the females in the same sample population in order to describe adolescent female dieters. They found that 60 percent of the 446 females had been on reducing diets by the time they became seniors in high school, a much larger percentage than could be objectively classified, from a medical standpoint, as in need of weight reduction. Dieting behavior was found to be related to not just body weight and/or body fatness, but also to characteristics of body build and a perception of overweight. Dieting practices differed from one weight group to another; obese dieters reported that they had started dieting at an earlier age, had stayed on diets longer, and had lost more weight on their diets than non-obese dieters.

Adolescence is a particularly important part of the life cycle in which to examine disordered eating behaviors and their precursors. It is a time in life when physical appearance becomes crucial, and carries a high emotional loading. The value of thinness is internalized at an early age in American society(14,15,16); by the early teen years there is a strongly felt need for peer acceptance along many dimensions, including that of body weight. An adolescent may indulge in unrealistic thinking, coming to believe that the rules that apply to everyone else do not pertain in this instance, and thus take terrible risks to achieve the physical ideal, i.e., thinness. The

anorexic believes unrealistically that, regardless of what they do to support their extreme thinness, their health will survive. Bulimics believe, equally unrealistically, that they can "have their cake and eat it, too."

METHODS

Construction of Instrument

A seventy-eight item self-administered questionnaire was constructed to elicit information about weight-associated behaviors, attitudes toward food preparation and eating, social circumstances under which eating occurs, and attitudes toward one's own physical appearance. It was also designed to assess relationships among weight-for-height, desired weight-for-height, and self-image. It was not designed for case finding.

The items were developed from personal clinical experience, a survey of the clinical literature, and from the diagnostic criteria for anorexia nervosa and bulimia as given in DSM III. This was to assure that the questionnaire would assess those eating behaviors and attitudes toward food and eating that are clearly disordered, and those that could be considered disordered if they were to cluster in certain ways. The diagnostic criteria are the following: for anorexia nervosa, (A)intense fear of becoming obese, which does not diminish as weight loss progresses, (B)disturbance of body image, (C)weight loss of at least 25 percent of original body weight, or if under 18 years of age, weight loss from original body weight plus projected weight gain expected from growth charts may be combined to make the 25 percent, (D)refusal to maintain body weight over a minimal normal weight for age and height, and (E)no known physical illness that would account for the weight loss. For bulimia, (A)recurrent episodes of binge eating (rapid consumption of a large amount of food in a discrete period of time, usually under two hours), (B)at least three of the following: (1)consumption of high caloric, easily ingested food during a binge, (2)inconspicuous eating during a binge, (3)termination of binges by abdominal pain, sleep, social interruption, or self-induced vomiting,(4)repeated attempts to lose weight by severely restrictive diets, self-induced vomiting, or use of cathartics or diuretics, (5)frequent weight fluctuations greater than ten pounds due to alternating binges and fasts, (C)awareness that the eating pattern is abnormal and fear of not being able to stop eating voluntarily, (D)depressed mood and self-deprecating thoughts following binges, and (E)bulimic episodes not due to anorexia nervosa or any known physical disorder.

The questionnaire was pretested and found to take approximately 30 minutes to complete.

Population Sample

Subjects for this research were 2276 suburban students between the ages of 13 and 18 (1298 females and 968 males). The suburban population represented in the study exhibits very low minority representation, and is predominently Northern and Eastern European in origin. Property values in these suburbs during the last decade, when compared with other parts of the metropolitan area, are an indication that the socioeconomic status of the population is middle to upper middle class. Subjects were concentrated in the 15-to-16 years age bracket because this appears to be the lower limit at which heightened risk for anorexia nervosa occurs(17). Breakdown by age is presented in Table I.

TABLE I
Age of Subjects

Age in Years	Number	Percent of Total
13	11	.5%
14	350	15.4%
15	1089	47.8%
16	667	29.3%
17	102	4.5%
18	31	1.4%

Twelve high schools participated in the survey. To approximate a representative sample of 15- to 16-year olds from each school, the questionnaire was administered in required health or physical education classes at the grade levels most appropriate for those ages. In every instance the administration of the questionnaire took place during the school day in a classroom setting. Students were instructed not to identify themselves by name or in any other manner on the questionnaire. They were assured that they would not be asked to participate in the study in any other way, and that filling out the questionnaire would not affect whatever grade they might be assigned in the class.

RESULTS

Weight, Height and Self Image

The self-reported weights, heights and ages of the 2147 students who reported all three characteristics were compared with standards established by the American Child Health Association as revised by T. D. Wood and B. T. Baldwin(18). They are reported in Table II as percents of standard. It can be seen that a higher percentage of females (37.7 percent) than males (24 percent) report weights and heights that place them at less than 95 percent of standard, while 31 percent of males and 24.9 percent of females report at something over 104 percent of standard.

Three males and one female reported weights that put them below 75 percent of standard, and an additional 26 males and 72 females fall in the 75 through 84 percent category. Thus, 4.8 percent of the sample is, by self-report, very thin.

A total of 101 males and 110 females reported weights that are in excess of 114 percent of standard for height and age. So 9.8 percent of the population is overweight or obese. No one reported a weight in excess of 150 percent of standard.

No attempt was made to measure weights and heights of subjects. Previous research has shown that obese adolescent girls may exhibit an underreporting bias, although the reported and actual weight were only a few pounds apart. Adolescent boys and non-obese girls exhibited no such bias (19). This study focuses upon attitudes and behaviors rather than physical characteristics.

The students were asked to indicate what had been happening to their weight since achieving their present height; had they been gaining, losing, or staying the same? The results are displayed in Table III. Marked differences are apparent in self-reported female and male experiences. A lower percentage of females than males at all weights expressed as a percent of standard reported that weight had increased since achieving present height. A higher percentage of females than males in four of the weight

TABLE II
Percent Weight for Height and Age According to Standard

	Male	Female	Row Totals
50 through 74 percent	N=3 % Males = .3 % Total = .1	N=1 % Females = .1 % Total = .01	4 (0.2%)
75 through 84 percent	N=26 % Males = 2.8 % Total = 1.2	N=72 % Females = 5.9 % Total = 3.4	98 (4.6%)
85 through 94 percent	N=194 % Males = 20.9 % Total = 9.0	N=386 % Females = 31.7 % Total = 18.0	580 (27.0%)
95 through 104 percent	N=386 % Males = 41.5 % Total = 18.0	N=446 % Females = 36.6 % Total 20.8	832 (38.8%)
105 through 114 percent	N=187 % Males = 20.1 % Total = 8.7	N=194 % Females = 15.9 % Total = 9.0	381 (17.7%)
115 through 124 percent	N=60 % Males = 6.5 % Total = 2.8	N=65 % Females = 5.3 % Total = 3.0	125 (5.8%)
125 through 149 percent	N=41 % Males = 4.4 % Total = 1.9	N=45 % Females = 3.7 % Total = 2.1	86 (4.0%)
Column Totals	N=930 (43.3%)	N=1217 (56.7%)	

TABLE III
What Has Happened to Weight Since Achieving
Present Height Expressed as Percents of Males and Female Totals

Present Weight for Height and Age According to Standard	Decreased		Remained the Same		Increased		Fluctuated	
	Males	Females	Males	Females	Males	Females	Males	Females
50 through 74 percent	0%	0%	0%	.1%	.3%	0%	0%	0%
75 through 84 percent	.1%	0%	1.2%	3.1%	1.3%	1.2%	.2%	1.6%
85 through 94 percent	.9%	1.8%	10.8%	17.1%	6.1%	3.8%	3.1%	9.2%
95 through 104 percent	.9%	2.9%	20.3%	14.8%	12.5%	5.8%	7.9%	14.1%
105 through 114 percent	1.5%	1.2%	9.5%	5.8%	6.1%	3.1%	3.0%	5.8%
115 through 124 percent	.3%	.7%	3.0%	2.9%	1.3%	.8%	1.8%	1.7%
125 through 149 percent	.2%	.5%	1.9%	1.0%	1.1%	.8%	1.1%	1.4%

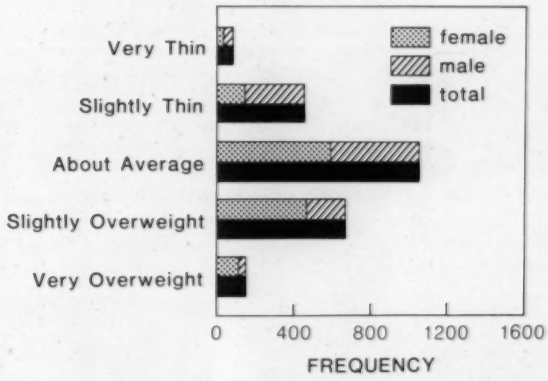
categories (two at the high end of the scale and two in the middle) reported decreasing weights. It is also apparent that, in every weight category except the two highest, approximately twice the percentage of females than males

reported fluctuating weights. The reports of weight loss and weight fluctuations did not correlate with reported experience of serious physical illness, serious mental illness, serious emotional problem, or death or other tragedy in the family.

When asked how they view themselves at their present weights, the students responded as follows: very thin, 2.5 percent; slightly thin, 19.0 percent; about average, 45.0 percent; slightly overweight, 28.8 percent; very overweight, 4.4 percent.

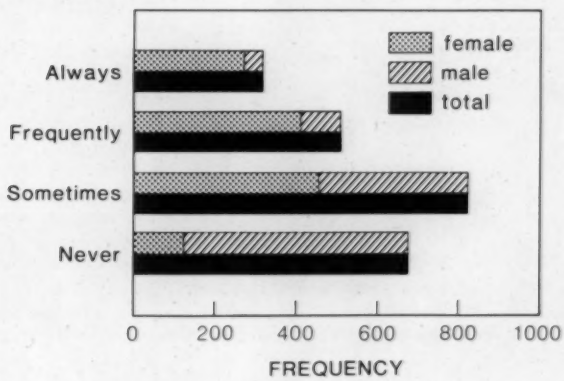
Figure I illustrates that a higher percentage of males than females view themselves as thin, and a higher percentage of females see themselves as overweight. Yet the comparison of reported weights, heights and ages with

FIG. I
View of Self at Present Weight



the standard showed that a higher percentage of females than males falls below 95 percent, and a lower percentage is above 114 percent. The differential cultural pressures on the sexes with respect to body weight are quite evident here, and become moreso upon examination of Figure II. The

FIG. II
Worry About Being or Becoming Overweight



students were asked to indicate the frequency with which they worry about being or becoming overweight. Those who always or frequently worry are preponderately female, those who sometimes worry are about evenly split between sexes, and those who never worry are preponderately male.

In an effort to understand what a preoccupation with weight might mean in terms of self-assessment of overall physical attractiveness, a forced-choice question was asked regarding feelings about one's own physical attractiveness. Most of the males and all of the females whose weight was above 124 percent of standard felt they were not physically attractive. Males in the other weight categories distributed themselves quite evenly across other possible responses to the question--as physically attractive as anyone, or physically attractive if pains are taken to appear so. The exception was the response, "I am more attractive than most people;" almost all males who chose this response were in the 95-through-114-percent-of-standard category. For females, there was an inverse relationship between feelings of attractiveness and percent weight according to standard that was significant at the .001 level. No females who reported feeling more attractive than most people fell above the 94-percent-of-standard-category.

It is interesting to look at what the students felt were ideal weights for themselves at the particular point in time (Table IV). Of those whose actual weights fell between 95 and 104 percent of standard, a higher percentage of males than females indicated an ideal weight that also fell between 95 and 104 percent of standard, about 15 percent of both sexes said they would like to weigh less, and about 7 percent of both sexes said they would like to weigh more. Higher percentages of females than males who fell below 95 percent of standard in reported weight also gave ideal weights that fell below 95 percent of standard. Most students who were overweight indicated ideal weights that would put them at or below standard weights for height, age and sex. It has already been shown that the more slender the adolescent female, the more likely it is that she will feel herself as attractive or more attractive than other people. Yet 94 percent of females who weigh between 75 and 84 percent of standard, and 57 percent who weigh between 85 and 94 percent of standard, indicated an ideal weight that is greater than reported weight.

Results of the Factor Analysis

Factor analysis is primarily a technique for data reduction. Factor-analytic techniques allow a researcher to see if some underlying pattern of relationships exist within an array of correlation coefficients for a set of variables. If such a pattern is found to exist, then the data can be reduced to a smaller set of factors. Such factors are interpreted as source variables which account for the observed interrelations in the data(20).

The factor analysis performed on the data in this study involved the following steps: [1]Sixty-nine variables were selected for the analysis. The selection was based on the judgment of three individuals who have had a great deal of clinical and research experience with a broad spectrum of eating disorders. Each read the questionnaire and selected those questions (variables) he or she felt could give an indication of disordered eating behavior, either alone or in clusters. [2]Product-moment correlation coefficients were calculated as a measure of association for the set of sixty-nine variables. [3]A new set of variables, called initial factors, was constructed on the basis of the correlation coefficients. These new variables were defined as exact mathematical transformations of the original data.

TABLE IV
Self-described Ideal Weight
Expressed as Percent Weight for Height and Age According to Standard

Indicated Weight Expressed as Percent Weight for Height and Age According to Standard	50 through 74 percent		75 through 84 percent		85 through 94 percent		95 through 104 percent		105 through 114 percent		115 through 124 percent		125 through 149 percent		150 through 174 percent		Row Total	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
50 through 74 percent	N=0	N=0	N=0	N=0	N=0	N=0	N=0	N=1	N=1 *100.0% ** .8%	N=0	N=0	N=0	N=0	N=0	N=0	N=0	N=1	N=1
75 through 84 percent	N=0	N=0	N=3 13.6% 6.2%	N=4 6.2% 6.3%	N=9 40.9% 3.2%	N=29 44.6% 6.8%	N=5 22.7% 1.6%	N=24 36.9% 6.0%	N=4 18.2% 3.2%	N=5 7.7% 3.5%	N=1 4.5% 2.7%	N=3 4.6% 5.2%	N=0	N=0	N=0	N=0	N=22	N=65
85 through 94 percent	N=0	N=1 .3%	N=6 3.3% 12.5%	N=16 4.4%	N=59 32.6% 21.2%	N=138 38.1% 32.6%	N=75 41.4% 23.4%	N=136 37.6% 34.0%	N=29 16.0% 23.4%	N=44 12.2% 30.6%	N=9 5.0% 24.3%	N=20 5.5% 34.5%	N=3 1.7% 30.0%	N=3 .8% 50.0%	N=0	N=4 1.1% 80.0%	N=181	N=312
95 through 104 percent	N=1 .3% 33.3%	N=1 .3%	N=21 5.9% 43.8%	N=22 5.6% 34.9%	N=114 32.2% 41.0%	N=147 37.2% 34.8%	N=147 41.5% 45.8%	N=147 37.2% 36.7%	N=47 13.3% 37.9%	N=55 13.9% 38.2%	N=14 4.0% 37.8%	N=22 5.6% 37.9%	N=4 1.1% 40.0%	N=1 .3% 16.7%	N=5 1.4% 71.4%	N=0	N=353	N=395
105 through 114 percent	N=2 1.1% 66.7%	N=0	N=12 6.8% 25.0%	N=11 6.2% 17.5%	N=61 34.7% 21.9%	N=71 39.9% 16.8%	N=57 32.4% 17.8%	N=59 33.1% 14.8%	N=31 17.6% 25.0%	N=28 15.7% 19.4%	N=10 5.7% 27.0%	N=8 4.5% 13.8%	N=2 1.1% 20.0%	N=1 .6% 16.7%	N=1 .6% 14.3%	N=0	N=176	N=178
115 through 124 percent	N=0	N=0	N=4 7.1% 8.3%	N=3 5.2% 4.7%	N=15 26.8% 5.4%	N=23 39.7% 5.4%	N=27 48.2% 8.4%	N=20 34.5% 5.0%	N=8 14.3% 6.4%	N=6 10.3% 4.2%	N=2 3.6% 5.4%	N=5 8.6% 8.6%	N=0	N=1 1.7% 16.7%	N=0	N=0	N=56	N=58
125 through 149 percent	N=0	N=0	N=2 5.1% 4.2%	N=7 16.7% 11.1%	N=20 51.3% 7.2%	N=15 35.7% 3.5%	N=10 25.6% 3.1%	N=13 31.0% 3.2%	N=4 10.3% 3.2%	N=6 14.3% 4.2%	N=1 2.6% 2.7%	N=0	N=1 2.6% 10.0%	N=0	N=1 2.6% 14.3%	N=1 2.4% 20.0%	N=29	N=42
Column Totals	N=3	N=2	N=48	N=63	N=278	N=423	N=321	N=400	N=124	N=144	N=37	N=58	N=10	N=6	N=7	N=5	N=828	N=1101

* Percent of Row Total
** Percent of Column Total

Twenty-one such orthogonal (i.e., independent) initial factors were extracted with eigenvalues greater than one. An eigenvalue is a special measure computed in the process of deriving a factor, and is a measure of the relative importance of the factor. By convention, factors with eigenvalues greater than one can be regarded as significant(21). [4]A principal-component analysis was performed in order to transform, or rotate, the twenty-one initial factors into a new set of principal components (terminal factors) that are not correlated with one another. Eight such terminal factors were defined with eigenvalues greater than one. Together these eight factors constitute the best linear combination of variables, that is, they account for more of the variance in the data as a whole than any other linear combinations of variables. Together the eight factors account for 65.7 percent of the variance (see Table V).

The regression coefficients of factors to describe a given variable are called factor loadings. The factor loading .30 was selected as the minimal loading acceptable in defining a terminal factor. Twenty-six of the variables had loadings of .30 or higher. Those variables, their loadings expressed as a percent, and the factors they define are presented in Table VI. Three variables (2,9, and 13) each load on two factors; hence, these are complex variables which are measuring more than one theoretical dimension.

It can be seen from the loadings that the factors can be quite easily interpreted. They were named as follows:

Factor 1: Preoccupation with Weight

This factor correlated with being female at the .001 level. It described a group of people who were not overweight, yet worried about being or becoming overweight. They were currently dieting to lose weight, felt they have weight problems, and had a view of themselves as being less attractive than most other people. Members of this group liked to be thin and liked to lose weight. They felt the need for increased exercise after eating and became angry with themselves after overeating. These attitudes and concerns were further manifested by weighing once daily or oftener, and by mentally reviewing one's food intake at the end of the day. This factor appears to represent a general preoccupation with weight and eating.

Factor 2: Physical Activity

There were high loadings on items concerned with participation in sports and exercise and with a view of oneself as more active in such endeavors than one's companions. This activity occurred in the absence of a great deal of concern about food and weight; it was not tied to an expressed need for exercise after eating. Factor 2 correlated significantly with being male (p=.001).

TABLE V

Results of the Factor Analysis of 69 Variables (N=2020)

Factor	Eigenvalue	Variance (%)	Cumulative (%)
1. Preoccupation with weight	4.82472	19.6	19.6
2. Physical activity	2.68671	10.9	30.5
3. Overweight	2.25505	9.2	39.7
4. Special foods	1.47270	6.0	45.6
5. Vomiting	1.37660	5.6	51.2
6. Amenorrhea	1.32032	5.4	56.6
7. Food avoidances	1.20079	4.9	61.5
8. Likes to cook	1.02928	4.2	65.7

TABLE VI

The Loadings of the Items on the 8 Factors*

	FACTORS	1	2	3	4	5	6	7	8
1.	Percent weight for height and age according to standard	-	-	-67	-	-	-	-	-
2.	Self View	-51	-	-65	-	-	-	-	-
3.	Worries about becoming/being overweight	67	-	-	-	-	-	-	-
4.	Weigh self daily or oftener	30	-	-	-	-	-	-	-
5.	Now dieting to lose weight	62	-	-	-	-	-	-	-
6.	I have seen a doctor for unexplained vomiting	-	-	-	-	47	-	-	-
7.	I have seen a doctor for being overweight	-	-	31	-	-	-	-	-
8.	I am now seeing a doctor for unexplained vomiting	-	-	-	-	74	-	-	-
9.	I have weight problems	36	-	45	-	-	-	-	-
10.	I enjoy preparing more than actually eating food	-	-	-	-	-	-	-	70
11.	I become angry with myself after overeating, no matter how often I do it	73	-	-	-	-	-	-	-
12.	I eat special foods or prepare foods in special ways in order to improve my health	-	-	-	87	-	-	-	-
13.	I avoid special foods or kinds of cooking in order to improve my health	-	-	-	33	-	-	37	-
14.	Members of my family eat special food or prepare foods in special ways in order to improve their health	-	-	-	48	-	-	-	-
15.	Members of my family avoid special foods or kinds of cooking in order to improve their health	-	-	-	-	-	-	32	-
16.	I participate in sports or exercise more than most of my companions	-	77	-	-	-	-	-	-
17.	I exercise - running, biking, situps, tennis, etc., at least daily	-	47	-	-	-	-	-	-
18.	Menstrual periods not regular? (females)	-	-	-	-	-	62	-	-
19.	Have stopped menstruating for several months	-	-	-	-	-	75	-	-
20.	I like to lose weight	55	-	-	-	-	-	-	-
21.	I like to be thin	59	-	-	-	-	-	-	-
22.	I cook for family or friends at least once a week	-	-	-	-	-	-	-	65
23.	I participate in more sports than most of my companions	-	82	-	-	-	-	-	-
24.	I am more active than most of my companions	-	54	-	-	-	-	-	-
25.	I feel a need for increased exercise after eating	52	-	-	-	-	-	-	-
26.	I often review the day's food intake at the end of the day	47	-	-	-	-	-	-	-

*Loadings are expressed as percentages. Only loadings 30 and greater are shown.

Factor 3: Overweight

The highest loadings (both negative) occurred on percent weight for height and age according to standard, which indicates a condition of overweight, and on self-view, which indicates a view of self as less attractive than other people. More moderate loadings occurred on items indicating that a physician had been consulted about the condition of overweight, and that there was an acknowledgment that a weight problem existed. This factor seems to represent the existence of overweight in the absence of drastic measures to try and remedy the situation.

Factor 4: Special Foods

The relevant items had to do with food choice behaviors and food preparation techniques. Certain foods and preparation techniques are selected in order to enhance health, while others are avoided. Other family members share these behaviors.

Factor 5: Vomiting

Only two items loaded on this factor. Both had to do with seeing a physician for treatment of unexplained vomiting. Items regarding self-induced vomiting and vomiting associated with eating did not show factor loadings of .30 or greater.

Factor 6: Amenorrhea

The items were "have stopped menstruating for several months" and "menstrual periods not regular." There was no loading on items which would indicate that these were associated with low body weight or with unusual attitudes or behaviors regarding food. It correlates, of course, with being female.

Factor 7: Food Avoidances

This factor is related to Factor 4. Both loaded on the food avoidance item. Factor 7 also loaded on the item which indicates that family members also practice food avoidances in the interest of health.

Factor 8: Likes to Cook

Loading occurred on items which indicated that cooking for others is very pleasurable, and that it is more pleasurable to cook than it is to eat. There was no loading on items which would have indicated an overriding preoccupation with food or weight.

It can be seen that the factor structure is dominated by the first factor; subsequent factors account for relatively small amounts of variance.

The reliability coefficient Cronbach's alpha was computed for each of the eight factors. The alphas for six of the factors range from .57 to .73; alphas for Factors 3 and 6 were uninterpretable due to statistical artifact. Reliability is defined as the variation over an indefinitely large number of independent repeated trials of errors of measurement(22). The advantage of using Cronbach's alpha as a measure of reliability over using a test-retest sample is that Cronbach's alpha calculates reliability in the absence of intervening events.

There were a number of linear combinations of variables which emerged as initial factors, but not as terminal factors, which are informative and interesting. One such initial factor loaded on the items, "knows how many

calories one consumes in a day" and "takes laxatives after eating too much." Another loaded on "takes laxatives regularly" and "has enemas regularly." A third loaded on the items "vomits after overeating" and "makes self vomit after overeating." A fourth loaded on "frequently overeat" and "frequently have times when I stuff myself." None of these correlated significantly with sex.

Three initial factors which correlated with being female (p=.001) were one which loaded on "has few friends" and "enjoys self most when alone," a second which loaded on "sometimes I hide food to make others think I have eaten more," and a third which loaded on "my family fights over food" and "meals with my family are generally unpleasant."

The technique of factor analysis has been utilized in two research projects involving anorexic patients(23,24). Both resulted in a test or an inventory designed for case identification and for assessment during a therapeutic process. In each case, the terminal factors which emerged resemble some of the eight factors described in this paper. The factors from the Fichter and Keeser research were: (1)figure consciousness; (2)feelings of insufficiency; (3)anancasm (obsessive-compulsive); (4)adverse effects of meals; (5)sexual anxieties and (6)bulimia. Factors from the Garner and Garfinkel research were: (1)food preoccupation; (2)body image for thinness; (3)vomiting and laxative abuse; (4)dieting; (5)slow eating; (6)clandestine eating; and (7)perceived social pressure to gain weight.

Prevalence of Self-Reported Behaviors and Attitudes Which Present a Potential for Eating Disorders

The frequencies of food- and weight-related behaviors and attitudes which may be indicative of problems with food and eating are displayed in Table VII. Striking differences in prevalence among females and males emerged for several of the variables. Although frequent overeating was reported by approximately one-third of the females and one-fourth of the males, self-anger associated with the behavior was reported by over two-thirds of the females and only one-fifth of the males. Almost one-half of the females but less than one-tenth of the males indicated that they were dieting at the time the questionnaire was administered. At the same time, thirty percent of the males but only three percent of the females were dieting to gain. Expressions of positive attitudes toward losing weight and being thin were three to four times more prevalent among females. Females were also much more likely to tie the felt need for exercise directly to eating. It is interesting that two of the most drastic and potentially harmful responses to overeating, the use of laxatives and the induction of vomiting, were about equally as prevalent in both sexes. It appears that somewhere between 3 and 5 percent of the adolescent population is engaging in these health-endangering practices. The sexes are also approximately the same in self-reported incidence of family fighting regarding food. About one-third of all families apparently use food, i.e., the amount or kind of food that members are or are not eating and the occasions at which eating occurs, as a modality for the expression of displeasure, anger, or frustration.

DISCUSSION

It has recently been established that the health of adolescent females is significantly related to their health in adulthood, and that the trait of

TABLE VII
Frequencies of Self-Reported Food- and Weight-Related Behaviors and Attitudes that May Present a Potential for Disorder

	% Females	% Males	% Total
Overeats frequently	34.1	27.5	30.8
Self-anger for overeating	68.9	21.4	47.9
Now dieting to lose	48.0	8.4	30.8
Now dieting to gain	3.0	30.4	14.6
Likes to lose weight	67.8	26.1	46.0
Likes to be thin	59.2	15.9	37.6
Hides food	8.3	6.5	7.4
Family fights over food	32.8	28.9	30.8
Induces vomiting after overeating	2.8	2.2	2.5
Uses laxatives after overeating	2.7	3.3	2.9
Needs exercise after eating	65.1	31.5	51.0

being satisfied with oneself in early adolescence is significantly related to health in adulthood for both meals and females(25). The data reported here suggest that a high proportion of adolescents are dissatisfied with their physical selves in terms of weight-for-height, and that adolescent females are differentially preoccupied with weight, food, and eating.

Bruch(26) has described a group of dieters which she calls "thin-fat." These are people who psychologically resemble patients with anorexia nervosa but who do not display the profound loss of weight. Factor 1 would seem to describe just such a situation--preoccupation with and concern about weight in the absence of an indication of a physical weight problem. It may originate in the apparent conflict which exists between biologically induced changes which occur in the female shape during adolescence and a culturally derived high positive value on extreme slenderness for females. This conflict, in addition to predisposing to serious eating disorders, may constitute a more subtle impediment to female development. For many females, the issue of control/loss of control may be dealt with in the context of food and weight, rather than in a context which would be psychologically and developmentally more sound. It is interesting that 50 young women in this study who had clearly achieved the cultural ideal in body weight, i.e., fell below standard weight for height, indicated as their ideal weights amounts that would clearly define them as overweight or obese. Perhaps for them the concept of ideal weight was the weight they would like to be in the absence of cultural stigmatization. Perhaps it is an indication of the onerous burden it may be to young women to achieve and maintain the cultural ideal.

The results of this study confirm that the concerns of American adolescents regarding body weight and physical attractiveness which were identified and described almost two decades ago(27) are still present. Females are more deeply involved than males in a general preoccupation with weight and eating. Self-anger and fear are much more pervasive among females than males as emotional responses to these facets of their lives. The increasing incidence of severe eating disorders among adolescent females may be in part a reflection of this. We have identified discrete subsets of the adolescent population in American society, characterized by differences in self-reported behaviors and attitudes with regard to food and eating. This may be useful for prospective research on eating disorders and other related problems.

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COMPARISON OF TWO METHODS TO EVALUATE NUTRIENT INTAKES OF FRENCH CANADIAN ADULTS

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ABSTRACT

In view of developing a short method of dietary analysis, multiple regression equations of nutritional intakes on food group ingestion frequencies were derived from 3-day dietary records of 204 males and 201 females French Canadian adults. For each sex, both complete and stepwise equations were calculated for energy, total protein, total fat, complex carbohydrate, concentrated sugar, alcohol, calcium, iron, retinol, thiamin, riboflavin, niacin and ascorbic acid. It was found that the stepwise procedure yielded equations with coefficients of determination (R^2) of the same order of magnitude as those of complete regression equations while decreasing considerably the number of food groups retained as predictors, and that predicted intakes were highly correlated with intakes calculated by a standard method. For 6 of the 13 dependent variables studied, the R^2 values show that 71% or more and 67% or more of the variation was explained by the stepwise equations for males and females respectively. Factors affecting the predictive value of the equations and means of improving it are discussed. The results are promising enough to justify further work toward the validation of a short method which would become an efficient tool in the hands of community nutritionists to evaluate the nutritional quality of diets of target groups before and after interventions.

KEY WORDS: Nutrient intake, adults, predicting equations, ingestion frequency.

INTRODUCTION

Standard methods used to evaluate the quality of diet are based on calculation of the nutritive value of every single food item qualitatively and quantitatively described in a dietary record. Such methods are tedious, time-consuming and require qualified personnel.

Some investigators have tried to develop shorter methods by which the intake of certain nutrients could be estimated from the frequency of ingestion of predictive food groups. To identify and score predictive groups for calories, protein, carbohydrate and fat, Heady (1) performed stepwise regression analyses with intakes calculated by a standard method as dependent variables; the indepen-

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dent variables were the ingestion frequencies of 134 food groups, each multiplied by a weighting factor based, in most cases, on the average serving size. Hankin et al. (2, 3) also performed stepwise regression analyses, using ingestion frequencies of average portions of 32 selected food groups to predict intakes of calories, protein, carbohydrate, fat and sodium. More recently, Hankin et al. (4) used the ingestion frequencies of 50 selected food groups plus squared age as independent variables to predict the intake of calories, protein, total fat, saturated fat, carbohydrate and cholesterol. The conclusion of those different authors is that their method, although promising, has not been satisfactorily validated. Moreover, the food habits of the population studied: British (1), Japanese American (2, 3) and Japanese Hawaiian (4), are quite different from those of French Canadians, so that the result could not be applied to the latter.

The present paper is a report of the first part of an ongoing study designed to develop and validate a short method of dietary analysis adapted to the French Canadian population, based on non-weighted frequencies for all-inclusive food groups, and allowing the estimation of calories, total protein, total fat, complex carbohydrate, concentrated sugar, alcohol, calcium, iron, retinol, thiamin, riboflavin, niacin and ascorbic acid. The key elements of the proposed method are multiple regression equations with high predictive value, and in which as few food groups as possible are retained so that the ingestion of a given food constituent can be predicted from information on a limited number of food groups. The precise purpose of this paper is to examine the predictive accuracy of the equations obtained in this initial step of the study. Observations on the nutritive quality of the diets of our subjects will be presented elsewhere.

METHODS

The data were collected between June 1978 and August 1980 from 405 volunteers, 204 males and 201 females aged 31 to 55 years. All subjects were of French Canadian descent and lived within 40 miles from Quebec City.

The average level of education and gross family income of our sample were indicative of a higher socio-economic status than that of the population of Quebec as a whole. Forty six percent of the men and 24% of the women had at least a college education, while the most recent corresponding figures for Quebec adults, those of 1976 (5, 6) were 25% and 19% respectively. Forty nine per cent of our subjects reported an annual gross family income of $30,000 or more, while that level was reached in 1978 by only 19% of Quebec families (7). However, the reported family incomes ranged from less than $10,000 to more than $100,000 per year, and the terminal levels of education from elementary classes to graduate school.

The dietary data were obtained in similar proportions (approximately 25 ± 2%) during the four trimesters of the year by seven trained individuals, either dietitians or dietetic technicians. Three-day measured food records including two week days and one weekend day were obtained from the subjects. The daily record was divided into 7 periods: night, breakfast, morning, dinner, afternoon, supper and evening. The subjects were seen twice, once to receive instructions, and the second time to verify the information given in the records. This information was then compiled by the standard method, using the table of nutritive values by Brault-Dubuc and Caron-Lahaie (8).

Food grouping

In order to elaborate the short dietary method, 62 groups of foods belonging to 11 categories and including all foods and beverages found in the subjects' dietary records have been constituted (Table 1). Two criteria were used for the

grouping: the similarity of nutritive values within one group and the food habits of French Canadians. The grouping was deliberately broad to take into account the customary inaccuracies of food records. A list of all specific foods to be included in each group, with appropriate instructions for coding, was prepared and used in the computation of ingestion frequencies (9). The listing for the groups belonging to the "breads and cereals" and "milk and milk products" categories is presented as example in Table 2.

TABLE 1

Food Categories and Food Groups[1]

BREADS and CEREALS
- white bread
- brown bread
- whole-grain cereals
- processed cereals
- crackers
- pasta

MILK and MILK PRODUCTS
- milk
- yogurt
- cheese

MAIN DISHES
- eggs
- liver and kidneys
- meat I (beef)
- meat II (pork)
- poultry
- fish
- shellfish
- combination foods I
- combination foods II
- white sauce dishes
- italian dishes
- specialty meats
- legumes

SAUCES
- white
- brown
- salad

VEGETABLES, COOKED
- potatoes
- french fried
- yellow or green I
- yellow or green II
- others
- pickles
- vegetable juices

VEGETABLES, RAW
- yellow or green I
- yellow or green II
- others

SOUPS
- cream soups
- others

FRUITS
- fruit drinks
- fruits I
- fruits II
- citrus fruit
- dried fruit
- nuts and seeds

DESSERTS
- cookies
- cakes
- pies
- frozen desserts
- milk desserts
- pancakes
- gelatin
- syrups

BEVERAGES
- hot with milk
- hot, black
- carbonated
- beer
- wine
- spiritous liquors

MISCELLANEOUS
- butter
- cream
- candies
- chips
- added sweets

[1] A list of specific foods to be included in each food group was prepared (see text and Table 2).

Computing ingestion frequencies

The data of the dietary records were transformed into ingestion frequencies of the different food groups for each of the 7 periods of the day. Within a particular period, as long as one food item of a group had been consumed, this food group was counted once, and once only, independently of the amount or of the number of items of the group that were consumed.

However, the group "added sweets" was an exception to the preceeding rule: one frequency was counted every time a food item of this group was eaten, even within the same period. For instance, a breakfast composed of two English

muffins with marmalade and of coffee with milk and sugar was tabulated in the following way: one frequency for the "white bread" group of the "breads and cereals" category, one frequency for the "hot beverage with milk" group of the "beverage" category and two frequencies for the "added sweets" group of the "miscellaneous" category, one for marmalade and one for sugar in the coffee.

TABLE 2

Lists of specific foods to be included in food groups of the "breads and cereals" and "milk and milk products" categories, with instructions for coding[1]

CATEGORIES/FOOD GROUPS	
Breads and cereals	
White bread	Rusk, biscuits, "grissol", melba toast, English muffins, raisin bread, white bread, cracked wheat bread, hamburger roll, hot dog roll
Brown bread	Whole wheat bread, rye bread, other whole-grain breads such as oatmeal bread, "bon matin" and "quotidien" breads
Whole-grain cereals	Shredded wheat, "croque-nature", granola, oatmeal, "Pep", bran. All include the milk. If pre-sweetened, or if sugar is added, count one frequency for "added sweets". A mixture of wheat germ and processed cereals belongs to this group.
Processed cereals	All processed breakfast cereals, such as: corn flakes, "Special K", etc. as well as cream of wheat and baby cereals. All include the milk. If pre-sweetened, or if sugar is added, count one frequency for "added sweets".
Crackers	All unsweetened items, garnished or not, such as cheese sticks, pretzels, soda crackers, "Escort", "Ritz", croutons, pop corn.
Pasta	All pasta, rice, semolina
Milk and milk products	
Milk	Used as drink, natural or flavored. For chocolate milk, count one frequency for "added sweets"
Yogurt	All types: natural, flavored, with fruit
Cheese	All varieties; fondue "parmesan", Swiss fondue. This group also includes cheese added to a dish; examples: cheese in cheeseburger, "coquilles St-Jacques", lasagna, cheese macaroni, parmesan on spaghetti, pizza, cheese sauce.

[1] Lists with appropriate coding instructions were prepared for each of the 62 food groups and used in the computation of ingestion frequencies (9).

The "cheese" group as well as the "dessert" category were also treated in a particular way. One frequency was attributed to the "cheese" group every time cheese was present in a dish unless it had already been recorded during the same period. Thus cauliflower "au gratin" induced one frequency for the group "others" of the "cooked vegetables" category and another one for the "cheese" group of the "milk and milk products" category. On the other hand, "added sweets" were never accounted for as one frequency when they accompanied any food of the "desserts" category. Therefore, a cake garnished with strawberry jam was inscribed in the "cake" group only.

Statistical analysis

SPSS programs (10) were used to perform multiple regression analyses. The dependent and independent variables respectively were the mean nutritive values for 3 days calculated by a standard method and the ingestion frequencies of the 62 food groups. The data of the male and female subjects were treated separately. At first, a complete regression equation was obtained, i.e. all the independent variables were retained in the equation. Then, a stepwise procedure was applied in which only the variables with F values of 1.34 or more ($P \leq 0.25$) were allowed to enter the equations. The purpose of the latter type of analysis is to yield an optimal prediction equation with as few predictor variables as possible.

RESULTS

Complete and stepwise multiple regression equations were calculated for energy and for 12 food constituents. Table 3 shows the equation derived from the stepwise regression analysis to predict the calcium intake of male subjects. Twenty one food groups out of 62 were retained in the equation, which predicts a calcium intake of 1712 mg for Subject 001 while the value calculated by the standard method was 1658 mg.

TABLE 3

Equation obtained by stepwise multiple regression analysis to predict calcium intake in males[1,2,3]

Mg calcium = 352.82 + 261.63 (cheese)[4] + 291.41 (milk) + 250.61 (whole-grain cereals) + 213.06 (yogurt) + 268.62 (cream soup) + 154.25 (processed cereals) + 64.19 (meat I) + 41.72 (hot beverages with milk) - 75.29 (candies) + 206.92 (syrup) + 200.26 (milk desserts) + 52.78 (fruits II) - 83.66 (potatoes) + 152.20 (legumes) - 37.15 (cookies) + 60.26 (fruit drinks) - 144.30 (Italian dishes) + 73.27 (cooked vegetables yellow or green I) - 80.05 (combination foods II) + 65.63 (wine) + 109.46 (pasta)

[1] Variables are listed in the order of entry into the equation

[2] All variables included had an individual F value of 1.34 or more ($P \leq 0.25$)

[3] The coefficient of determination of the equation is 0.72 and the equation is significant at $P \leq 0.005$

[4] Ingestion frequency of the food group mentioned in parenthesis.

Different reasons why a given independent variable can be retained as a predicting group are illustrated by that equation; thus, among the food groups retained as predictors of calcium intake for the male subjects of our sample,

- cheese, milk, yogurt, cream soups, hot beverages with milk, milk desserts were obviously, to different degrees, actual sources of calcium;
- other groups may have been associated with important sources of calcium, although they do not themselves contain appreciable amounts of that element; this is particularly evident for syrups, wine and pasta;
- still others were associated with a low consumption of calcium since they enter the equation with a negative coefficient: candies, potatoes, cookies,

combination foods, and also Italian dishes in spite of the fact that they often contain cheese.

Since this paper is a report on the first step of an ongoing study, the composition of all the regression equations will not be presented.

The coefficient of determination (R^2) of a multiple regression equation represents the percent of the total variance accounted for by the equation and is used to evaluate its prediction accuracy. In Table 4, for each sex, two series of coefficients of determination are shown, corresponding respectively to the complete and the stepwise regression models. The R^2's ranged from 0.89 to 0.51 and from 0.88 to 0.43 for the complete and stepwise analyses respectively. It is important to note that the F values for the equations corresponding to the R^2's presented in Table 4 were all significant at the $P \leq 0.005$ level.

Depending on the food constituent concerned, the stepwise procedure retained between 15 and 35 food groups. The R^2 values for the stepwise regression equations are lower than for the complete regression equations; however, the decrease is small in most cases. The only R^2 which shows a decrease greater than 0.05 is that of thiamin for the female subjects. Moreover, for 6 variables out of 13, the coefficients of determination show that the stepwise equations account for 71% or more of the variation for males, and 67% or more for females. Five of those 6 variables are the same for both sexes, namely concentrated sugar, alcohol, retinol, riboflavin and ascorbic acid, whereas calcium and complex carbohydrate are the sixth one for males and females respectively.

DISCUSSION

The present findings, using a stepwise regression analysis of nutritive values on food ingestion frequencies, demonstrate that 1) equations with coefficients of determination of the same order of magnitude as those of complete regression equations are obtained while decreasing considerably the number of food groups retained as predictors and 2) predicted intakes are highly correlated with intakes calculated by a standard method. It is the first time, to our knowledge, that such results are reported for several vitamins and minerals as well as for energy and macronutrients and without using weighting factors to take into account the average size of servings.

Moreover, the present results are based on a greater number of subjects, 204 males and 201 females, than those of similar studies: 97 males (1), 73 males (2) and 53 females(4), and on dietary data collected over all four seasons of the year, a fact which is not reported by other workers (1, 2, 4). Compared to that of subjects previously studied, the age span of our subjects, 31-55 for both sexes, is wider than that of men who were 40-55 years old (1) and 35-55 years old (2) but narrower than that of women whose age varied approximately from 40 to 70 ($X \pm S.E. = 57.4 \pm 0.7$) (4). The socio-economic status of the present sample, reflected by a wide range of levels of education and family incomes, is in all likelihood much more varied than that of the population of bank clerks studies by Heady (1) whereas Hankin et al. (2, 4) do not characterize the socio-economic status of their subjects.

The coefficients of determination obtained by stepwise regression analysis in those previous studies (1, 2, 4) are higher than ours for the same food constituents and for subjects of the same sex. The R^2's obtained in this study for men and women respectively are 0.51 and 0.61 for energy, 0.50 and 0.56 for total protein and 0.49 and 0.55 for total fat, while the corresponding R^2 values are 0.67, 0.57 and 0.73 respectively for Japanese American men (2), and 0.88, 0.72 and 0.81 respectively for Japanese Hawaiian women (4). In the latter study, squared age is introduced as an independent variable and is retained, with a negative coefficient, in the equations for calories, total fat and saturated fat.

TABLE 4

Coefficients of determination (R^2) for equations obtained with a complete or a stepwise multiple regression analysis[1,2]

Dependent variable	Men (N=204) No. of independent variables	R^2	Women (N=201) No. of independent variables	R^2
Energy	62[3]	0.57	62	0.65
	33[4]	0.53	26	0.61
Protein	62	0.54	62	0.61
	27	0.50	20	0.56
Fat	62	0.53	62	0.57
	24	0.49	27	0.55
Complex carbohydrate	62	0.66	62	0.69
	27	0.62	29	0.67
Concentrated sugar	62	0.77	62	0.72
	22	0.74	28	0.70
Alcohol	62	0.87	62	0.89
	17	0.86	22	0.88
Calcium	62	0.75	62	0.68
	21	0.72	22	0.64
Iron	62	0.59	62	0.58
	35	0.57	22	0.53
Retinol	62	0.74	62	0.76
	25	0.71	25	0.74
Thiamin	62	0.56	62	0.51
	20	0.51	15	0.43
Riboflavin	62	0.74	62	0.70
	28	0.72	31	0.68
Niacin	62	0.65	62	0.60
	31	0.63	32	0.59
Ascorbic acid	62	0.79	62	0.70
	21	0.77	21	0.68

1 The coefficient of determination is the percentage of the variance of the dependent variable accounted for by the independent variables included in the equation

2 All the equations were significant at the 0.005 level according to a Fisher test

3 Total number of independent variables included in all analyses; this number of variables was retained in the equations obtained by the complete regression analysis

4 Number of independent variables in the equations obtained by a stepwise analysis where only variables with an F Value of 1.34 or more ($P \leq 0.25$) were allowed to enter the equation.

The only R^2's reported by Heady (1), working with a male British population, are 0.77 and 0.72 respectively for animal fat and for vegetable plus marine fat.

The large number of subjects certainly contributes to the obtention of R^2's as significant for the stepwise regression models as for complete models along with a substantial diminution in the number of independent variables. However, the large size and the socio-economic heterogeneity of the sample, as well as the variation in food patterns associated with the seasonal factor, introduce a greater variance in our data than in those of studies done by the previous authors (1, 2, 4), and lower the coefficients of determination while increasing their significance. Moreover, it must be suspected that higher coefficients could be obtained by including non-food variables in the regression model, as did Hankin et al. (4).

It is important to consider the perspective in which our food grouping is done in respect to that used in similar studies. In fact, previous workers are interested in testing hypotheses on the relationship of food intake to the incidence of cardiovascular disease (1, 2) or cancer (4). As a result of these specific objectives, their interests are limited to the prediction of calories, total protein, fat and carbohydrate, and, in some cases, sub-categories of fat (1, 4) sugar (1), cholesterol (4) and sodium (2), totally ignoring all vitamins and minerals other than sodium. Logically, then, their main criterion for grouping foods is a similarity as sources of the few food constituents studied, thus allowing much more homogeneity within groups than is the case in the present study where we take into account the vitamin and mineral content as well. This might explain in good part the higher R^2's obtained in those more limited studies. Moreover, a lesser degree of precision is deliberately accepted in not using a weighting factor for the ingestion frequencies (1, 2, 4), in order to eliminate the necessity of obtaining quantitative estimates of food intake when using the proposed method. In that perspective, the coefficients of determination obtained in the present study might be considered satisfying. However, they vary from 0.43 to 0.88 for different food constituents (Table 2), and this variation within the results of the present study may be explained by several factors.

A first explanation might be that the R^2 value is influenced by the variation in the amount of the predicted constituent present in different food items within the same predicting group. Thus, a small variation of the amount of alcohol within its important predicting groups, namely "beer", "wine" and "spiritous liquors": most beers contain 5% alcohol, wines 12% and liquors 40%, might explain the high coefficients of the alcohol equations, 0.86 for males and 0.88 for females. On the contrary, an important variation of the quantity of fat exists within some of its predicting groups. In fact, it is well known that the fat content varies greatly between kinds and cuts of meat. Since one of the predicting groups for fat is the "meat I" group, which includes lamb, beef, veal, horse-meat, brains, heart, roe-deer, moose, tongue, sweetbread and smoked meat, this might partially explain the week R^2's, 0.49 for males and 0.55 for females, observed for fat.

Secondly, the prediction value of the regression equation is likely to be greater if an important quantity of the predicted constituent is present in a limited number of predicting groups. The prediction of alcohol, for which high R^2's are obtained, clearly illustrates this point, since alcohol is found almost exclusively in three groups: "beer", "wine" and "spiritous liquors". In contrast, when a nutrient is present only in moderate amounts in several groups, the precision of the prediction decreases. For example, the three following categories: "main dishes", "breads and cereals" and "milk and milk products", include 22 food groups that are important sources of protein. It is then not surprising to obtain relatively low R^2's for protein: 0.50 for males and 0.56 for females respectively.

A third explanation could be that the difference between the lowest and the highest quantities ingested by the subjects influences the prediction value: a

wider range will allow for a better prediction. Thus, the mean daily alcohol consumption of the male subjects of our sample varied between 0 and 177 gm of pure alcohol, and the R^2 is high (0.86). On the other hand, the relatively small range of thiamin ingestion by the females of our sample (0.41 to 2.57 mg) reduces the prediction value, giving a R^2 of 0.43.

Finally, the prediction is undoubtedly influenced by the precision of the measure of the independent variables. Now, that precision is not equally influenced for all food groups by the use of non-weighted frequencies. For instance, in many cases, one frequency for "beer" will represent one 341 - ml bottle. In contrast, the size of portions for "main dishes" varies greatly. For example, one frequency for steak might represent 60 gm for one individual and 300 gm for another, both being seen as one serving. This may explain, at least partially, why the prediction for energy, protein, fat and iron, which is influenced by several important predicting groups of the "main dishes" category, is less precise than that of other constituents: all of them have R^2's equal or inferior to 0.61 for both males and females.

One or more of the above explanations may apply to the prediction accuracy of a given equation. Thus, the high precision of alcohol prediction seems to be associated with the constant quantity of the predicted constituent in a small number of predicting groups, to the large differences in the quantities ingested, and to the fact that the utilization of ingestion frequencies does not greatly affect the precision of the measure.

The precision that can be considered acceptable for a short method of dietary analysis depends on the proposed use of the method. Higher coefficients of determination are needed in order to predict the nutritional intake of individuals, which was the purpose of the studies by Hankin et al. (2, 4) than to classify the elements of a population into groups according to the quality of their diet, which was the objective pursued by Heady (1). The purpose of our work is one of the latter type and in this respect, we have tentatively examined, for each of the nutritional variables studied, the degree of agreement between two classifications of the subjects: one done according to the calculated intakes obtained by the standard method, the other according to the predicted intakes from the stepwise regression equations discussed in this paper. The comparison was made by means of 3 X 3 contingency tables based on a division of the calculated values at the 33th and 66th centiles. Depending on the food constituent, the percentage of subjects identically classified by the two methods varied from 59.3 to 79.0 for men and from 55.2 to 76.1 for women. Very few subjects are classified with a two-degree disagreement, i.e. in the first class by one method and in the third by the other, or inversely (unpublished results). Although preliminary, these findings indicate the feasibility of using a short method of dietary analysis to differentiate sub-groups of a population in terms of the quality of their diet.

Further work is however needed before the short method of dietary analysis presented here can be put into use. The validity of our regression equations is currently being tested by applying them to dietary records of another group of subjects, and they will be modified if indicated in order to increase their predictive accuracy. Eventual modifications might involve: 1) the revision of the food grouping; for instance, rearrangement of the foods in the "main dishes" category to ensure a greater similarity of food values and thus increase the predictive value of the equations for calories, protein, fat and iron; 2) the addition of non-food independent variables, such as age, income and educational level; and 3) the analysis of each equation in respect to food groups that contribute to the prediction through association rather than as appreciable sources of the constituent concerned. Also equations suitable for other age groups: children, adolescents, elderly, will be worked out.

When satisfactory equations are in hand, the operations needed to estimate the dietary intake of subjects will consist of: 1) obtaining a dietary record where foods and beverages are broadly described, without the necessity of giving an accurate (or any) account of the quantity ingested; 2) transposing the information into ingestion frequencies of food groups and 3) applying the appropriate regression equation to predict the intake of calories and of each constituent.

The main advantage of the method is a simplification of the operations, resulting in a significative difference in the amount of time required to analyze the dietary records and thus reducing the cost of food surveys and the time lag before results are available. Moreover, the participation of the subjects being less laborious, the probability of obtaining a good cooperation is increased. This short method, then, when validated, will hopefully become an efficient tool in the hands of community nutritionists to evaluate the nutritional quality of diets of target groups before and after interventions.

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PHAGOCYTE FUNCTION IN PROTEIN-CALORIE MALNUTRITION

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ABSTRACT

Particle ingestion rate by phagocytes from malnourished children was adequate. Nitoblue tetrazolium (NBT) reduction was generally higher in children with kwashiorkor or marasmic kwashiorkor than in marasmic and well fed control children. There was no correlation between NBT reduction and bactericidal activity. The bactericidal activity of phagocytes was impaired, that of children with marasmic kwashiorkor being the most depressed. The mean leukocyte iodination value was higher in well fed children than in any of the malnourished groups. There was a significant relationship between quantitative iodination and bactericidal activity. The observed defective leukocyte iodination by phagocytes of malnourished children may be one of the factors responsible for impaired bactericidal activity and the latter is likely to be one of the causes of the high incidence of infections in these children.

KEY WORDS: Phagocytosis, protein-calorie malnutrition, protein deficiency, kwashiorkor.

INTRODUCTION

Several reports from different parts of the world where infantile malnutrition is prevalent have indicated impaired host defense mechanisms (1). The defects involve secretory antibody responses, cell-mediated immunity, complement system, the proportion and function of circulating thymus-derived T lymphocytes, antigen clearance by macrophages and serum siderophilin levels. Recently we (2,3) observed the presence of sheep erythrocyte rosette inhibitory substance in the sera of many malnourished children.

Impaired bactericidal capacity of leukocytes in malnutrition has been reported in many investigations (4-7) but normal results also have been observed (8). Studies carried out on small number of subjects, failure to separate the various conditions of nutritional stress, the frequent presence of concomitant infection and low sensitivity of some of the techniques are factors that could account for these discrepancies. In the present study, particle ingestion rates, nitroblue tetrazolium (NBT) test, bactericidal activity and quantitative leukocyte iodination were investigated in malnourished and well fed control children after minimising most of the problems stated above. The NBT reduction and quantitative leukocyte iodination were each correlated with bactericidal activity.

MATERIALS AND METHODS

Patients: The ingestion rate investigations were performed on 49 malnourished children. Using the classification recommended by the Wellcome Trust sponsored International Working Group (9), 17 were diagnosed as having marasmus, 24 kwashiorkor and 8 marasmic kwashiorkor. For bactericidal activity the study population consisted of 39 marasmic children, 44 with kwashiorkor and 44 with marasmic-kwashiorkor. Of the children investigated for bactericidal activity of their leukocytes, 16 children with marasmus, 19 with kwashiorkor and 9 with marasmic kwashiorkor had quantitative leukocyte iodination tests performed on their white cells. Also the leukocytes of 17 marasmic children, 24 children with kwashiorkor and 8 children with marasmic kwashiorkor who were tested for bactericidal activities also had NBT reduction tests performed on them.

The malnourished children came mainly from Ibadan and environs to the General Out Patients Clinic from where they were referred to the Paediatric Gastroenterology/Nutrition Clinics at the University College Hospital (UCH), Ibadan. The children were aged between 1-4 years.

Included in the leukocyte ingestion rate, NBT tests, bactericidal activity and quantitative iodination studies respectively were 18, 18, 37 and 12 age matched well fed control children (patient controls) from the same community as the malnourished children and attending the same Paediatric Clinic for minor ailments. Also included in the studies on bactericidal activity were 21 healthy children of workers at the University College Hospital Ibadan (healthy controls).

Assays: The ingestion rate and nitroblue tetrazolium tests were performed using the spectorphotometric method as described by Stossell (10). The plating method of Quie et al. (11) was employed in the measurement of bactericidal activity. The leukocyte iodination tests were performed using the method of Pincus and Klebanoff (12).

RESULTS

The marasmic children had the highest mean phagocytic ingestion rate and the widest scatter of values. The lowest ingestion rate for this group of children was 6.0 mg and the highest 25.6 mg of ingested paraffin oil per minute. The range in the control children was between 6.5 and 17.1 mg per minute while that of children with kwashiorkor and marasmic kwashiorkor was from 5.0 to 20.1 mg and 7.5 to 14.0 mg per minute respectively. The children with marasmic kwashiorkor had the smallest range. There were, however, no significant differences ($P>0.2$) between the mean phagocytic ingestion rates of the control and malnourished children (Fig. 1).

The mean NBT reduction rate was slightly higher in children with kwashiorkor and marasmic kwashiorkor than in the well fed control children. The values in the marasmic children were similar to those of the control children (Fig. 2). The NBT reduction rate by the leukocytes ranged between 132 and 523 µg per min in the control children and between 124 and 146 µg per min in the marasmic children. In the children with kwashiorkor the values ranged between 141 and 486 µg per min while the children with marasmic kwashiorkor had values ranging between 141 and 406 µg per min. The NBT reduction values were generally higher in the children with kwashiorkor or marasmic kwashiorkor than in the marasmic children and controls.

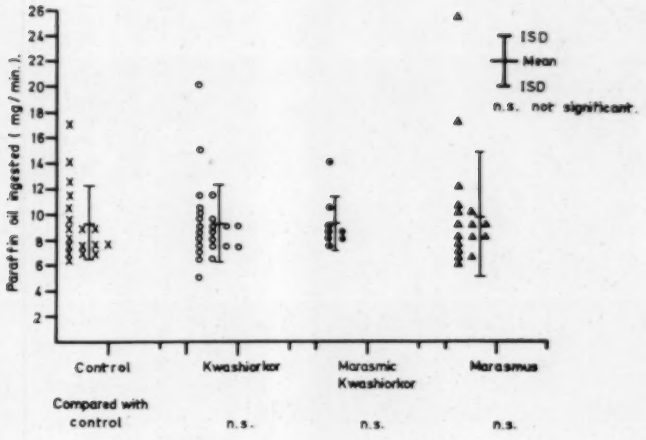

FIG. 1.
Ingestion rate of phagocytes

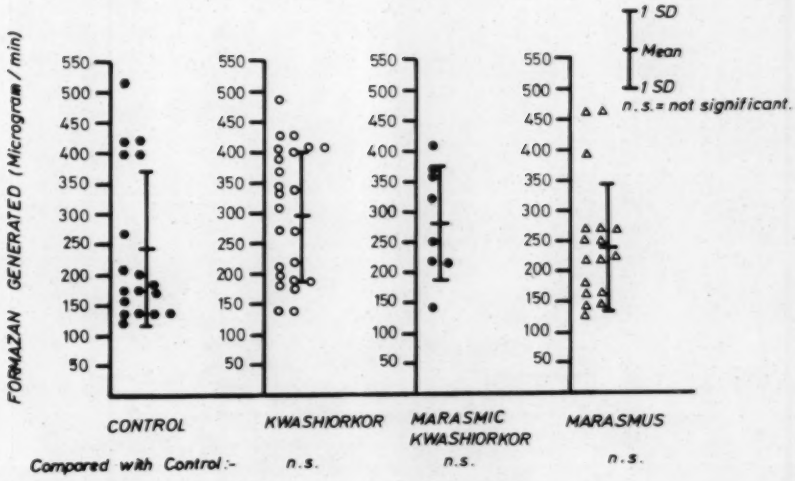

FIG. 2.
Nitroblue tetrazolium reduction by polymorphs

In the children with kwashiorkor the values ranged between 141 and 486 µg per min while the children with marasmic kwashiorkor had values ranging between 141 and 406 µg per min. The NBT reduction values were generally higher in the children with kwashiorkor or marasmic kwashiorkor than in the marasmic children and controls (Fig. 2).

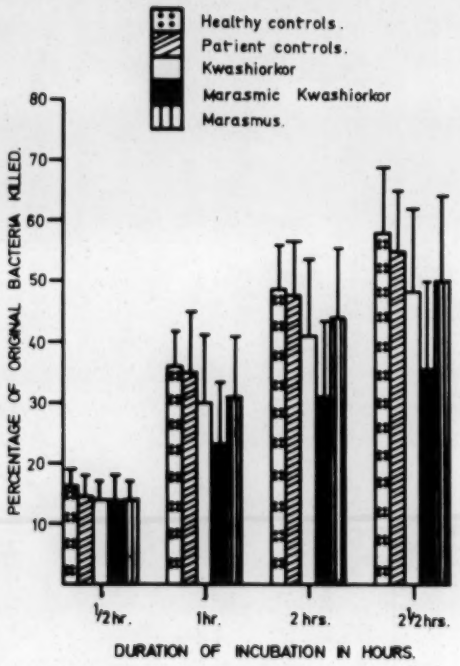

FIG. 3
Bactericidal capacity.

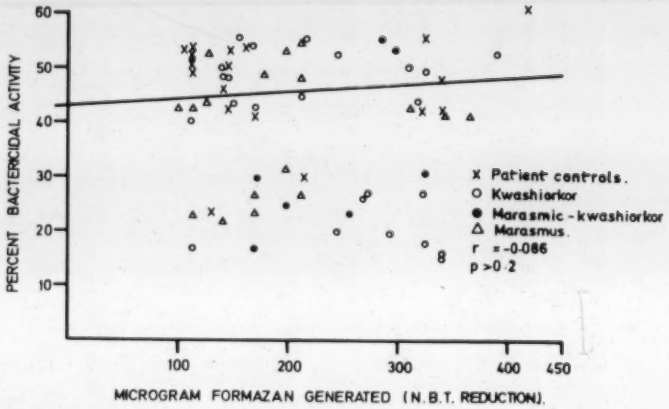

FIG. 4.
Correlation between NBT test and bactericidal capacity.

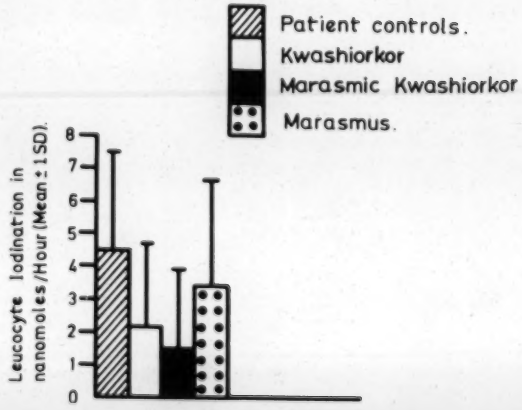

FIG. 5.
Iodination test.

The mean percentage bactericidal activities of the various study groups are shown in Fig. 3. For each group, the mean percentage bactericidal activity was lowest at 30 min and increased progressively up to 2 hr. Children having marasmic kwashiorkor showed the least rise with time. Children with kwashiorkor and those with marasmus appeared to differ only very slightly. There were no significant differences between the control and malnourished groups in the mean leukocyte bactericidal activity. At 2½ hrs children with kwashiorkor as well as those with marasmus demonstrated depressed bactericidal activity. The mean percentage bactericidal activity of the marasmic children was significantly different from that of the healthy controls at 1 hour and 2½ hours but not at 2 hours (Table 1). The reason for this isolated discrepant observation is not clear.

TABLE 1

Statistical Comparisons Between the Mean Percentage Bactericidal Activity of Phagocytes of Healthy Control Children and Those of the Malnourished Children

		Patient Controls	Marasmus	Kwashiorkor	Marasmic Kwashiorkor
Comparing the means of 30 min	t	1.2469	1.9437	1.9639	2.0020
	p	>0.2	>0.1	>0.1	>0.1
Comparing the means at 1 hr	t	0.5336	2.6147	3.1730	6.5398
	p	>0.2	<0.02	<0.01	<0.01
Comparing the means at 2 hrs	t	0.4566	1.9191	3.2268	7.0843
	p	>0.2	>0.1	<0.01	<0.01
Comparing the means at 2½ hrs	t	0.9398	2.3330	3.1697	6.9559
	p	>0.2	<0.05	<0.01	<0.01

The two control groups showed no significant differences in the mean bacterial killing capacities throughout the 2½ hour incubation period of the study.

A plot of the NBT reduction rates of leukocytes of the control and malnourished children against their corresponding bactericidal activities (Fig. 4) indicated the absence of an association between the two tests ($r = -0.086$, $p>0.2$).

The mean leukocyte iodination value was highest in the control children and lowest in children having marasmic kwashiorkor. The mean value of the former is about three times that of the latter (Fig. 5). The children with kwashiorkor had mean levels that were in between the mean levels of the children with marasmic kwashiorkor and the control children. Differences between the control children and the children with kwashiorkor or marasmic kwashiorkor are significant ($t = 2.2240$, $p<0.05$ and $t = 2.4870$, $p<0.05$ respectively). The mean leukocyte iodination in the marasmic children was only slightly lower than that of the control children ($t = 0.9467$, $p>0.2$).

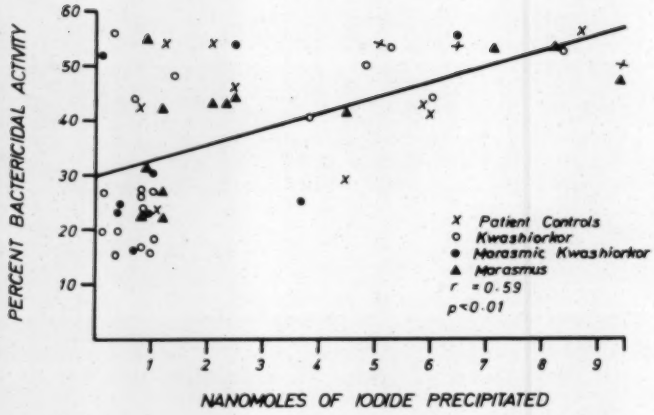

FIG. 6.
Correlation between iodination and bactericidal capacity.

The leukocyte iodination results were correlated with the corresponding bactericidal activities of the leukocytes. There was close relationship ($r = 0.59$, $p<0.01$) between the two tests (Fig. 6). It was observed in 21 out of 26 cases that children whose leukocyte iodination values were less than 1.2 nanomoles per hour also had defective bactericidal activities of their leukocytes. Except in 4 cases, leukocyte iodination values greater than 1.2 nanomoles corresponded with adequate leukocyte bactericidal activities.

DISCUSSION

Engulfment and destruction of bacteria by phagocytes of malnourished children have been extensively studied (3-7). The present study demonstrated normal ingestion rates by phagocytes of malnourished children. The findings corroborate observations in malnourished adults by Balch and Spencer (13). These workers employed morphological methods, i.e. counting the number of particles engulfed by the phagocytes. Results from the present study are also consistent with the observations in malnourished children by Seth and Chandra (4). In children with protein-calorie malnutrition, Selvaraj and Bhat (14) observed significantly lower phagocytic stimulation of hexose monophosphate shunt activity, a process linked with particle uptake. Cottigham and Mills (15) using morphological methods demonstrated decreased phagocytic activity of leukocytes in malnutrition. Presence of varying amounts of concomitant infections could be one of the major factors responsible for the observed defects in phagocytosis. The present studies demonstrate that ingestion which is the first stage in the destruction of microorganisms by phagocytes is normal in protein-calorie malnutrition.

Adequate reduction of NBT by leukocytes of children with marasmus and kwashiorkor observed in this study confirms other reports (16-18). This is an indirect evidence of efficient hydrogen peroxide production by the leukocytes (19) of malnourished children. On the other hand, decreased NBT reduction by leukocytes of malnourished children was observed by Kendall and Nolan (20) and Shousa and Kamel (21). Furthermore, Shilotri (22) showed by direct measurement that hydrogen peroxide production by leukocytes was considerably diminished in the malnourished children, whilst Selvaraj and Bhat (5) demonstrated diminished granule-bound reduced nicotinamide adenine dinucleotide phosphate (NADPH)

oxidase activity in leukocytes of malnourished children. Such discrepancies in results make findings difficult to interpret. It is logical to explain the conflicting observations on the problems stated above. In the present studies, efforts were made to minimise these problems by separating the malnourished children into 3 groups: kwashiorkor, marasmus and marasmic-kwashiorkor. A large number of malnourished and well fed control children were investigated. A sensitive spectrophotometric method was employed in the measurements of ingestion rate and NBT reduction by leukocytes.

The bactericidal activity of phagocytes of malnourished children was observed to be impaired in this study. The marasmic group only showed subtle defects in their bactericidal activities even after prolonged incubation. On the other hand, the bactericidal capacity of children having marasmic kwashiorkor was grossly impaired. Our findings confirm the observations in kwashiorkor by Douglas and Schopfer (7) who investigated only 5 malnourished and 5 control children. It was also observed in the present studies that in children with kwashiorkor the more severe the protein-calorie deficiency, the lower the bactericidal activity. These findings demonstrate the importance of quick detection and management of protein-calorie malnutrition. The observed impaired antimicrobial activity in malnourished children is likely to be one of the major causes of the higher incidence of infections that have been reported in these children by several workers (23,24).

There are many possible reasons for the impairment of bactericidal activities observed in kwashiorkor. It is known that for bacterial killing inside the phagocyte, several systems are required, e.g. the oxygen independent systems which include lactic acid (25) and lysosomal bactericidal agents (26). The lactic acid content of phagocytes in malnutrition has been reported to be diminished (27). It is doubtful, however, whether the deficiency of such a weak acid in malnutrition could, on its own, affect microbial killing so adversely as demonstrated in this study. Its presence may, on the other hand, be important for the activities of the other bactericidal agents. For instance, its absence or deficiency would depress the production of hydrogen peroxide by NADPH oxidase (28). Some workers have shown that cytoplasmic hydrolytic enzymes of leukocytes in protein-calorie malnutrition are present in adequate quantities (17,18,29) whereas others report reduced quantities (5).

The other important bactericidal system is oxygen dependent. It is now well established that following phagocytosis in mammalian neutrophils there is marked increase in oxygen utilization. This results in the production by NADPH or NADH oxidase, of superoxide anions and hydrogen peroxide (30) which have important antimicrobial functions (31). NADPH oxidase and mycloperoxidase have been observed to be depleted in the phagocytes of malnourished children (28).

Leukocyte iodination values in malnourished children were observed to be low. These findings corroborate the observations of Jose et al (45). The leukocyte iodination values correlated closely with the bactericidal activities. Assuming that the postulations of Klebanoff (33) and Pincus and Klebanoff (12) that leukocyte iodination tests are measures of the activities of the myeloperoxidase - hydrogen peroxide - halide complex are correct, our findings of low iodination tests in the white cells of malnourished children would indicate impairment in the activities of this complex.

Normal NBT reduction by leukocytes of malnourished children observed in this study suggests that there is likely to be adequate production of hydrogen peroxide. Although no studies to date have examined halide concentrations in malnourished children, levels of halides required for these activities have been reported to be low (28). The possibility of a deficiency of halides being one of the causes of reduced bactericidal activity can, however, not be ruled

out. It is also possible that the defect in bactericidal activity is causally related to low myeloperoxidase activity or combined myeloperoxidase-halide deficiency. Previous studies by Higashi et al. (34) have implicated myeloperoxidase depression for the decreased bactericidal activities demonstrated in iron deficiency patients. Peroxidase enzyme levels were, however, reported to be normal in the granulocytes of marasmic and kwashiorkor children (17).

It is likely that, despite normal concentrations of myeloperoxidase, hydrogen peroxide and halide, the conditions inside the cytoplasm of the phagocytes are not ideal for the efficient functioning of the complex. For instance, optimal activity of reduced NADH oxidase requires a pH of 5.5 (28). Presence of sufficient amount of lactic acid is, therefore, essential for maximal production of hydrogen peroxide.

In conclusion, the present studies have demonstrated that in protein-calorie malnutrition the phagocyte ingestion rate is normal. So also is the NBT reduction rate. The second stage which involves actual killing and digestion of the microorganisms is, however, impaired. The observed defect in the bactericidal activity of the leukocytes could be as a result of impairment in the oxygen-dependent system of phagocytosis.

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BLOOD PRESSURE IN YOUNG ADULTS AS ASSOCIATED WITH DIETARY HABITS, BODY CONFORMATION, AND HAIR ELEMENT CONCENTRATIONS.

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ABSTRACT

Clemson University students were studied to determine the role of anthropometric characteristics, types of physical activities, diet, and hair mineral concentrations on blood pressures. Hair was analyzed for Pb, Cd, Ca, Mg, Cu, and Zn by atomic absorption spectrophotometry. Higher blood pressures were associated with increased age, height, weight, and weight-height index. Sports activity was positively correlated with pulse pressures. Coffee consumption had a positive correlation with diastolic pressures and a negative correlation with pulse pressures. Males had greater blood pressures than females. Greater hair zinc, calcium, magnesium, cadmium, and cadmium to zinc ratio were associated with lower blood pressures. Greater hair lead concentrations were associated with higher blood pressures. Preference for lean meat, sweets, pork, poultry, and fruits and vegetables were associated with lower lower blood pressures. Those who preferred salt items had greater blood pressures.

KEY WORDS: Blood pressure, nutrition survey, minerals, anthropometric data.

INTRODUCTION

South Carolina often has the highest mortality rate in the nation due to cardiovascular disease (CVD) (1). Hypertension is a confirmed risk factor for this disease. Age, height, weight, and weight/height index have been associated with blood pressure in young adults (2).

Several minerals have been implicated in the development of hypertension. Schroeder (3) demonstrated that renal cadmium concentrations were significantly greater in subjects dying from hypertensive complications than in those dying of accidents or atherosclerotic heart disease. Dally et al., (4) found no difference in blood cadimum concentrations between hypertensives and normotensives but hypertensives had increased hair cadmium concentrations and decreased hair sodium concentrations. Low copper and high zinc concentrations in the diet can produce hypercholesterolemia in rats (5).

Previous studies in our laboratory on young adolescents demonstrated associations of hair minerals with blood pressures (6,7).

The objectives of this study were to examine the role of anthropometric characteristics, diet, and hair mineral concentrations on the blood pressures of a young adult population.

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MATERIALS AND METHODS

The study was conducted in the summer of 1980 at Clemson University during two summer school registrations. Most of the young adults studied ranged in age from 18 to 30 years, with less than 5% of the subjects above 30 years of age. A consent form was required of each participant. Blood pressure was recorded from the right arm in the sitting position with an electronic blood pressure instrument (Clark International, Inc., Milwaukee, Wisconsin). This instrument was able to measure pressure to the nearest mm Hg. Two trained emergency medical technicians and nurses measured blood pressures at tables separated from external influences. Height and weight were recorded for all participants to the nearest half inch and half pound. A questionnaire designed to evaluate food preference and time spent in various activities was self-administered by participants (Table 1). Subjects had hair samples removed from the nape of the neck. Stainless steel scissors were used to remove hair and scissors were washed between rinses in 95% ethanol. Hair samples were placed in envelopes and weighed 0.1 to 0.2 gm.

All chemical reagents were purchased from Fisher Scientific Co., Fair Lawn, New Jersey. All rinsings in the hair washing procedure used distilled deionized water. The washing procedure was carried out as follows. Hair samples were placed on moistened Whatman filter paper number 1 in a Buchner funnel. About 50 ml of water was passed over the hair sample and drained. Fifty milliliters of 1% sodium lauryl sulfate was added to the sample and drained. Suction was applied to remove suds. Two rinses with about 50 ml water followed. The remaining moisture was removed by suction. Hair samples were dried in a vacuum oven with 30 mmHg at 30°C (about 48 hours).

Weighed hair samples were placed in a glass beaker and digested and ashed in 5 ml of reagent ACS nitric acid (69-71%) and 1 ml of 70% perchloric acid (8). Samples were diluted up to 10 ml with water and stored in Nalgene plastic bottles (Nalge Co., Rochester, N.Y.).

For calcium and magnesium analysis, a 0.25% $SrCl_2$ solution was used as a diluting medium (9).

An atomic absorption spectrophotometer (Hitachi 170-50 AAS, Hitachi Corporation, Tokyo, Japan) was used for mineral analysis. Flame was used to analyze for Ca, Mg, Zn, and Cu. A graphite furnace (Hitachi GA-2 Graphite Atomizer, Tokyo, Japan) was used to analyze for Cd and Pb.

Standards were prepared from Certified Atomic Absorption standards. Proper background corrections were made. Hair mineral data was expressed as parts per million (ppm).

Blood pressure differences by sex were calculated by analysis of variance. The effect of weight, height, and weight/height index was determined by linear regression. Analysis of covariance was used to adjust for age, height, and weight effects upon sex. Multiple regression techniques were used to adjust for age effects upon height, weight, and weight/height index. Hair mineral data was fitted to linear, quadratic, cubic, or quartic expressions for best fit (10).

RESULTS

A sample size of 350 was obtained. Only three blacks participated, and thus race differences were not analyzed.

Age was positively correlated with diastolic pressure. Age was negatively correlated with pulse pressure. Height, weight, and weight/height were positively correlated with systolic, diastolic, and pulse pressures (Table 2). Hours per week spent on physical activity (sports) were positively correlated with pulse pressures. Coffee consumption was positively correlated with diastolic and negatively correlated with pulse pressures. Nonphysical activities, time spent watching television, number of breakfasts eaten per week, cola consumption, and cigarette smoking were not correlated with blood pressures.

Males had significantly greater blood pressures than females before and after covariant adjustment (Table 3).

TABLE 1

Questionnaire used in diet study at Clemson University

I. General Data - Part 1
Sex: Male_____ Female_____
Race: Black_____ White_____ Other_____

II. Physical Measurements
Blood Pressure Sitting: Systolic_____ Diastolic_____
Height_____

Please answer the following questions:

III. General Data - Part II
Age in Years:_____
County of Residence (if not a resident of South Carolina indicate state of residence)_____

IV. Activities: Indicate how many hours per week you spend on the following activities:
A. Physical Sports (jogging, competitive sports, hiking, etc.)
hours/week_____
B. Studying and pursuit of hobbies not involving physical exertion (Cards, sewing, reading, checkers, etc.)
hours/week_____
C. Television watching,
hours/week_____

V. Dietary habits
A. How many times per week do you eat breakfast?_____
B. How many cups of coffee do you drink per day?_____
C. How many bottles of cola do you drink per day?_____
D. Do you smoke?_____ If yes, packs per day_____
E. Taste Preferences: Check the space which corresponds to your opinion of the following food types:

	Dislike	Like Moderately	Like Much
Salty foods	_____	_____	_____
Sweet foods & desserts	_____	_____	_____
Spicy foods	_____	_____	_____

F. List the snack foods you eat
_____
_____
G. List the meats you eat
_____
_____
H. List the vegetables you eat
_____
_____
I. Do you trim fat from your meat? Yes_____ No_____

TABLE 2

Coefficients of Determination (r^2) of Blood Pressures by Items in Clemson Study*

Item	Systolic	Diastolic	Pulse
Age	NS[1]	.086(+)[2]	.031(-)
Height (inches)	.239(+)	.074(+)	.102(+)
Weight (pounds)	.292(+)	.151(+)	.082(+)
Weight/Height	.246(+)	.147(+)	.058(+)
Sports (hrs./wk.)	NS	NS	.058(+)
Nonphysical activity (hrs./wk.)	NS	NS	NS
TV Watching (hrs./wk.)	NS	NS	NS
Breakfast (times/wk.)	NS	NS	NS
Coffee Consumption (cups/day)	NS	.015(+)	.013(-)
Cola Consumption (bottles/day)	NS	NS	NS
Cigarettes (packs/day)	NS	NS	NS

[1]NS denotes no statistical significance ($p > 0.05$).

[2](+) or (-) denotes positive or negative correlation.

TABLE 3

Mean Blood Pressures by Sex, Unadjusted and Adjusted for Age, Height, and Weight in Clemson Study[1]

	N	Unadjusted			Adjusted		
		Systolic	Diastolic	Pulse	Systolic[2]	Diastolic[3]	Pulse[4]
Male	240	130a	72a	59a	128a	71a	59a
Female	115	112b	64b	48b	116b	66b	48b

[1]Means followed by different letters are statistically significant ($p<0.05$).

[2]Weight had a significant effect ($p<0.05$) on systolic blood pressures. Means are adjusted for weight effect.

[3]Age and weight had significant effects ($p<0.05$) on diastolic blood pressures. Means are adjusted for age and weight effects.

[4]Age had a significant effect ($p<0.05$) on pulse blood pressures. Means are adjusted for age effects.

Blood pressures were positively correlated with height, weight, and weight/height after age adjustment (Table 4). Pulse pressures were positively correlated with sports after age adjustment.

TABLE 4

Coefficients of Determination (R^2) of Blood Pressure by Height, Weight, Wt/Ht, Sports, Nonphysical Activity Corrected for Age by Multiple Regression in Clemson Study[1]

Item	Systolic	Diastolic	Pulse
Height (inches)	0.240(+)	0.154(+)[3]	0.137(+)
Weight (pounds)	0.293(+)	0.210(+)	0.129(+)
Wt/Ht	0.246(+)	0.204(+)	0.104(+)
Sports (hrs./wk.)	NS[2]	NS	0.048(+)
Nonphysical Activities (hrs./wk.)	NS	NS	NS

[1] R^2 value denotes combined effect of item and age upon blood pressure.

[2] NS denotes no statistically significant effect of age upon the item of interest ($p<0.05$).

[3] (+) denotes positive correlation.

Participants who indicated they disliked sweets and desserts had higher systolic and diastolic pressures than those who gave a much like rating for similar items (Table 5). Those who gave a like moderately rating to sweets and desserts had greater diastolic pressure than those with a like much rating. Participants who indicated they ate sweets and fruit and vegetable type snacks had lower systolic and diastolic blood pressures (Table 6). Those who preferred salt snacks had greater diastolic pressures than those who did not. Lower systolic pressures were observed for those who ate pork and poultry meats and trimmed fat from their meat. Greater diastolic and pulse pressures were noted for those who ate lamb or cured meats respectively.

Hair zinc concentrations were negatively correlated with systolic and pulse pressures; however, diastolic pressures increased as the zinc concentrations increased to about 125 ppm and then decreased thereafter (Figure 1). Calcium and magnesium concentrations were negatively correlated with systolic and diastolic pressures (Figures 2 and 3 respectively). Cadmium concentrations were negatively correlated with diastolic pressures (Figure 4). Lead concentrations correlated with diastolic pressures, first showing a modest decrease to about 20 ppm and then a modest increase to 32 ppm; however, the pulse pressures increased with greater lead concentrations (Figure 5). The cadmium to zinc ratio was generally negatively correlated with diastolic pressures (Figure 6).

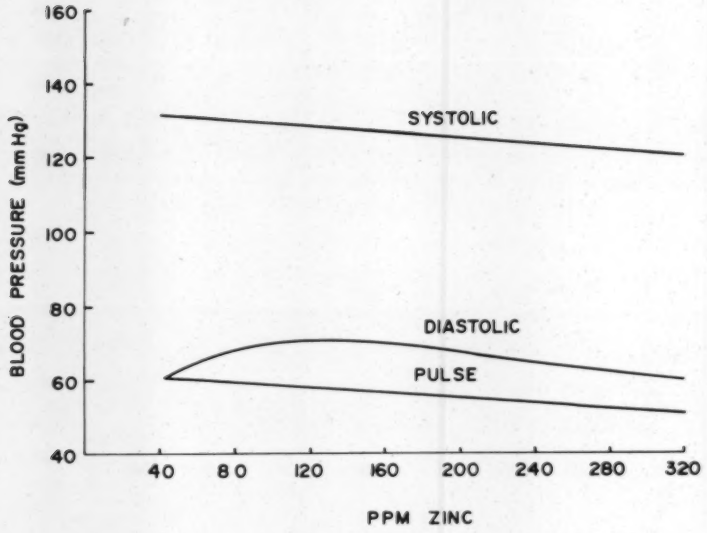

FIG. 1

Regression plots for the correlation of hair zinc concenrations with systolic, diastolic, and pulse pressures in Clemson study. Systolic R^2 = 0.042. Diastolic R^2 = 0.032. Pulse R^2 = .023.

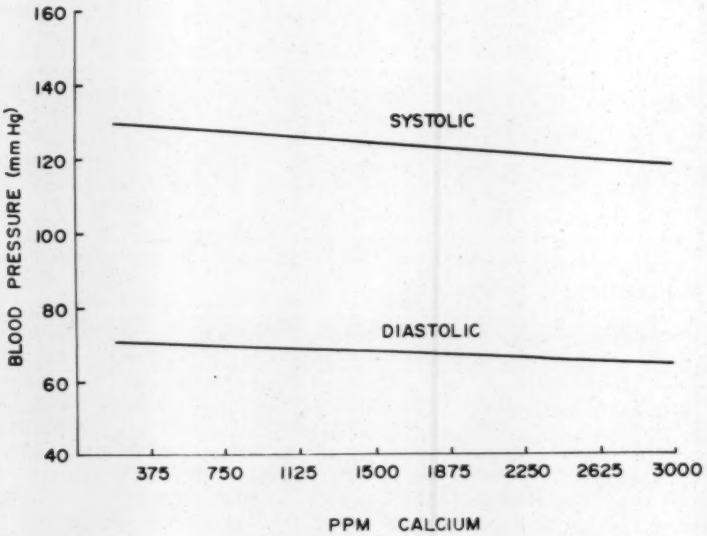

FIG. 2

Regression plots for the correlation of hair calcium concentrations with systolic and diastolic pressures in the Clemson study. Systolic R^2 = 0.026. Diastolic R^2 = 0.017.

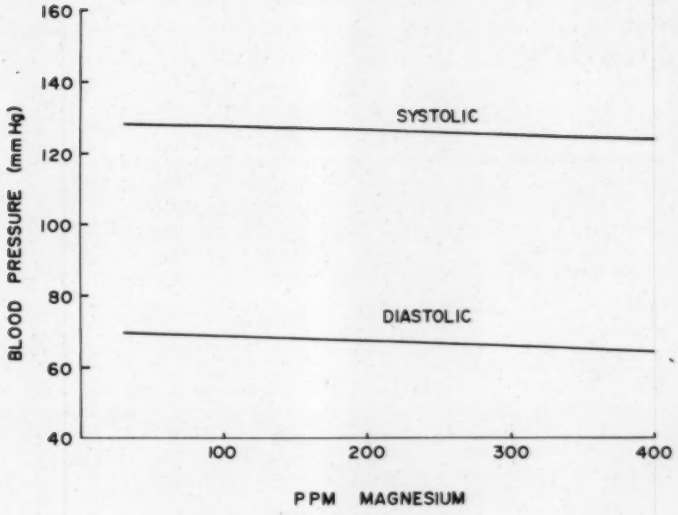

FIG. 3

Regression plots for the correlation of hair magnesium concentrations with systolic and diastolic pressures in the Clemson study. Systolic R^2 = 0.016.

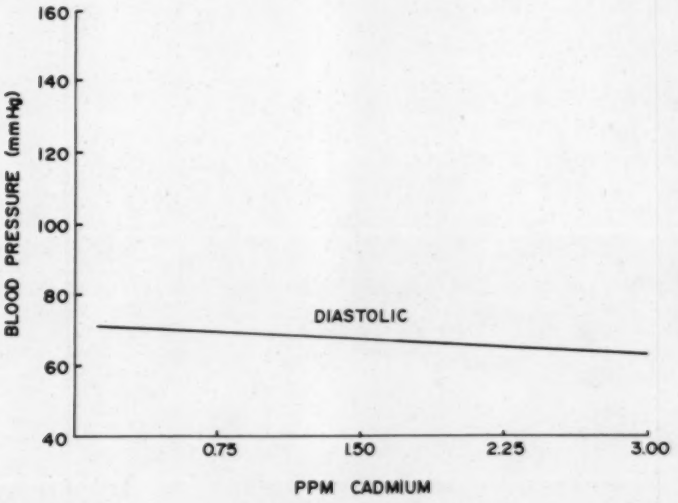

FIG. 4

Regression plot for the correlation of hair cadmium concentrations with diastolic pressures in the Clemson study. Diastolic R^2 = 0.015.

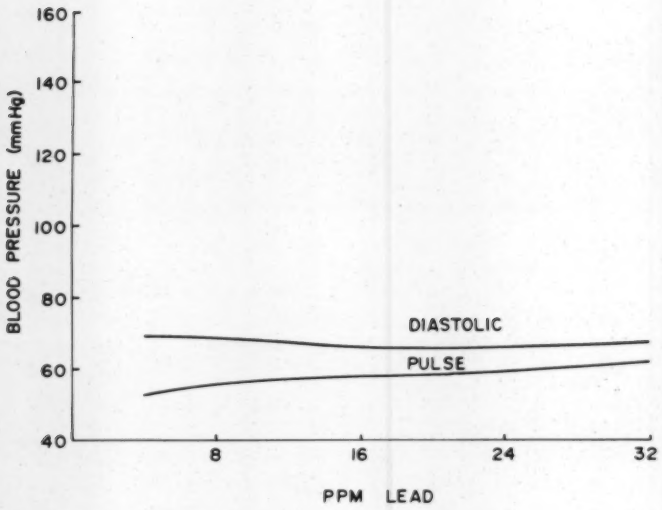

FIG. 5

Regression plots for the correlation of hair lead concentrations with diastolic and pulse pressures in the Clemson study. Diastolic R^2 = 0.020. Pulse R^2 = 0.018.

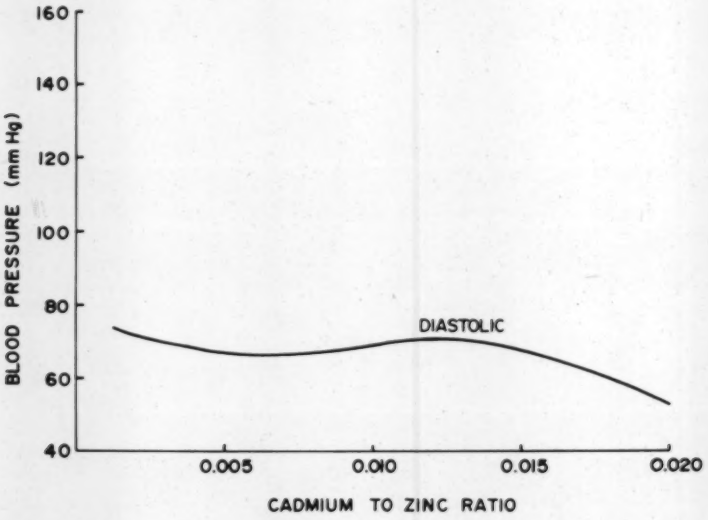

FIG. 6

Regression plot for the correlation of hair Cd/Zn ratio with diastolic pressures in the Clemson study. Diastolic R^2 = 0.029.

TABLE 5

Mean Blood Pressures by Preference for Sweets and Desserts in Clemson Study[1]

	N	Systolic	Diastolic	Pulse
Dislike	38	128a	71a	57
Like Moderately	204	125ab	70a	55
Like Much	96	121b	67b	55

[1]Means followed by different letters are statistically different ($p<0.05$). Duncan's Multiple Range test used.

TABLE 6

Mean Blood Pressures by Food Class Preference in Clemson Study[1,2]

Class Preference	N	Systolic	Diastolic	Pulse
SWEET SNACKS:				
Not eaten	168	126a	71a	NS[3]
Eaten	161	122b	67b	
SALT SNACKS:				
Not eaten	131	NS	67a	NS
Eaten	198		71b	
FRUIT OR VEGETABLE SNACKS:				
Not eaten	240	125a	70a	NS
Eaten	89	121b	67b	
PORK:				
Not eaten	48	127a	NS	NS
Eaten	286	124b		
POULTRY:				
Not Eaten	120	127a	NS	NS
Eaten	214	123b		
HAM, BACON, SAUSAGE:				
Not Eaten	277	NS	NS	54a
Eaten	57			58b
LAMB:				
Not Eaten	326	NS	69a	NS
Eaten	8		77b	
TRIM FAT FROM MEAT:				
No	91	128a	NS	NS
Yes	242	123b		

[1]Food consumption was not quantitative and may be a preference on the part of the participant in response to questionnaire (Table 1).

[2]Means followed by different letters are statistically different ($p<0.05$). Duncan's Multiple Range Test used.

[3]NS denotes no statistical significance ($p<0.05$).

There was a decrease, followed by a slight increase, and then by a decrease as the cadmium to zinc ratio increased (Figure 6). Copper and copper to zinc ratios were not related to any of the pressures.

DISCUSSION

Our results suggest that the weight/height index is the largest factor studied associated with blood pressures. Obesity as a factor in hypertension has been demonstrated on many different age groups (11-14). Stamler et al., (15) demonstrated that among Americans, the incidence of hypertension for overweight persons aged 20 to 39 years was double that of normal weight and triple that of underweight persons.

Stamler et al., (11) demonstrated that an increase in age was significantly related to systolic blood pressure. Our study did not show this, but that only diastolic and pulse pressures were correlated with age. Their study examined a larger population, blacks and whites, and an older age group. These factors probably explain the difference observed.

Mineral status and blood pressures have been investigated in several studies. Certain elements may be antagonist for one another or perhaps influence blood pressure in a complex manner (16-20). In the present study, higher hair concentrations of zinc, calcium and magnesium were associated with lower blood pressures. Here, the hair copper to zinc ratio was not related to blood pressures; however, in a rat study an increase in zinc relative to copper produced hypercholesterolemia (5), a finding which might suggest a hypertensive effect. In human hair, the authors noted that the diastolic pressures decreased with increasing hair cadmium to zinc ratio. This would be contrary to the finding by others of increased cadmium to zinc in kidneys of individuals succumbing to hypertension and its complications (3).

Hair minerals have been associated with disease states. Lower hair chromium, magnesium, zinc, and manganese are found in diabetics (21, 22). Hypothyroid patients have been reported to have increased Zn/Cu ratios in their hair. Decreased hair calcium concentrations are found in hyperlipidemic patients. Increased hair cadmium and decreased sodium concentrations have been demonstrated in hypertensives (22). Decreased hair copper, iron, and zinc concentrations are lower in malnourished black and white adolescents compared to healthy children of both sexes (23).

Evaluation of r^2 values for hair mineral data appear to indicate a weak association with blood pressures. Some minerals may affect the blood pressures of only a small percent of the population studied. Minerals may interact to produce an effect. Our study suggests that anthropometric data are larger factors in blood pressure levels than hair mineral concentrations. Further studies on the relationship of hair minerals to blood pressures are encouraged on larger sample sizes, since only a small fraction of a population's blood pressure may be affected by minerals.

Our diet study suggests the preference for sweets is associated with lower blood pressure. An explanation for this may be due to the weight of the individuals. Obese subjects rate sucrose solutions of strong concentrations as unpleasant compared to normal weight individuals (24). It is unknown whether this was the case in our study. Association of lower blood pressures with a preference for fruits and vegetables may be related to the potassium concentrations in those items. Elevated serum potassium concentrations are thought to be antihypertensive (25). High blood pressures were noted for those who preferred salt snacks and cured meats, which are high in NaCl. A high sodium diet can lead to hypertension (26-29). An explanation for the association of lower systolic pressures with pork and poultry remains unknown.

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TRANSPORT OF MACROMOLECULES IN MALNOURISHED ANIMALS
I. Evidence for Increased Uptake of Intestinal Antigens

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ABSTRACT

The uptake of tritiated bovine serum albumin (^{3}H)BSA from the small intestine into the portal circulation was studied in malnourished rats. Different concentrations were given and the quantity recovered in the serum was measured by gel filtration. The malnourished rats transported approximately twice that of the controls per g body weight at each level of protein given. These results suggest increased uptake of antigens in malnutrition.
KEY WORDS: Intestinal permeability, malnutrition, antigen transport

INTRODUCTION

Severe malnutrition is known to cause small intestinal mucosal atrophy (1,2,3) and altered intestinal mucosal defense (4,5,6). Based on these observations it has been suggested that the intestine of malnourished animals may become more permeable to intact or antigenically active protein fragments which, when taken up into the systemic circulation, could result in potentially adverse effects (7,8,9).

Chandra (9) reported an increase in circulating food antibodies in Indian children with severe malnutrition, suggesting that food antigens had crossed the intestinal barrier and evoked a systemic immune response. Worthington (7) gave rats bovine serum albumin (BSA) orally in proportion to body weight and found greater quantities of BSA in the sera of the malnourished animals compared to age-matched controls.

One of the difficulties in studying the uptake of intact proteins in malnourished and healthy animals is how to determine what are comparable amounts of protein to give when there is an appreciable difference in size between groups. Ideally, the amount should be directly proportional to the intestinal surface area of the animal. Most workers have assumed that this is equivalent to the intestinal weight of the animal which is known to be directly porportional to body weight. However, this has never been demonstrated. Furthermore, there is evidence that the relationship of gut weight to body weight is not the same in malnutrition as in health. Steiner, et. al. (10) have shown that in animals fasted for 6 days the weight loss of the small intestine was porportionally greater than total body weight loss.

In the following study, varying levels of BSA were given to malnourished and control rats to investigate not only whether there was increased uptake of antigen in malnutrition but also whether this phenomenon was dependent on the quantity of protein given. The results were expressed both in terms of body weight and, as an approximate indicator of intestinal surface area, in terms of intestinal DNA content.

MATERIAL AND METHODS

Male Sprague-Dawley rats, 4 weeks old and weighing 100-125 g, were used (Charles River Breeding Laboratories, Wilmington, MA). Animals were housed 4 per cage and initially fed ad libitum on Purina Rat Chow, a diet known to be free of BSA, (Ralston Purina Co., St. Louis, MO) for 3 days. They were then fed either the experimental low protein (0.5% lactalbumin) or control diet (18% lactalbumin) (Teklad Test Diets, Madison, WI) as described by Edozien (11) for 4 weeks. Water was allowed ad libitum.

Lyophilized crystalline bovine serum albumin (Sigma Chemical Co., St. Louis, MO) was radiolabeled with tritium using the Wilzbach reaction (12) (New England Nuclear, Boston, MA). The (^{3}H)BSA was then dialyzed in a phosphate buffered saline (PBS, 0.15M NaCl, 6.7mM NaH_2PO_4, 1.7 mM $NaHPO_4$, pH 7.4) solution and concentrated using a Minicon concentrator (Amicon Corp., Lexington, MA). Dialysis was found to be effective in removing free tritium and small molecular weight fragments from the albumin. This was demonstrated by applying a sample of the dialyzed and concentrated (^{3}H)BSA to a P-60 polyacrylamide column (Biorad, Richmond, CA). It was noted that greater than 92% of the radioactivity eluted in the void volume (MW>60,000). A trace amount of this was then added to crystalline BSA made up to a final concentration of 10, 100 or 300 mg per ml in PBS.

Animals were fasted for 16 hours before the experiment at which time they were anesthetized with ether. A midline incision was made, a ligature was placed around the ileum just proximal to the cecum, and the stomach located and lifted so that the pylorus was visible. While gently clamping across the pylorus to prevent backflow, 1 ml containing 10, 100 or 300 mg of unlabeled BSA with (^{3}H)BSA added as a tracer (specific activity = 2.3 - 4.6 x 10^5 cpm/mg) was slowly injected into the duodenum followed by 0.5 ml of air. The BSA was injected directly into the duodenum to minimize differences between groups in gastric digestion and emptying. The abdomen was then closed. Four hours later the animals were anesthetized, the midline incision reopened and blood collected from the portal vein. It has previously been established in our laboratory that the maximum concentration of serum BSA occurs 4 hours after its administration (13). The blood was centrifuged and the supernatant serum saved for analysis.

The total radioactivity recovered in the serum was calculated by multiplying the amount in a 0.05 ml sample by the total plasma volume of the animal. Plasma volumes were determined earlier in a comparable group of both malnourished and control animals by an isotope dilution method as described by Brooks (14).

The entire small intestine was removed, weighed and divided into 2 equal segments. Each half was rinsed with 10 ml of cold PBS and then homogenized in 10 ml of PBS and the total volume recorded. Everting of the malnourished intestine and scraping the mucosa with a glass slide was difficult because of its extreme friability. For that reason, the whole intestine was used. The DNA and protein content were measured according to previously described methods (15,16).

All samples were analyzed for tritium using a liquid scintillation counter (Mark II, Nuclear Chicago, IL). An aliquot of serum, 0.05 ml, was added to 10 ml of scintillation fluid (Aquasol, New England Nuclear, Boston, MA).

Concentrations of circulating BSA were determined by gel chromatography using a P-60 polyacrylamide column. This method, which measured partially degraded as well as intact BSA, was chosen because it has been previously reported that BSA fragments are antigenically active (17). Earlier studies, in which only intact BSA was measured, may have underestimated the potential antigenicity of the administered dose (7). The radioactivity in the void volume was assumed to represent intact or large molecular weight (LMW) fragments of BSA. The amount of BSA recovered via the portal system was estimated from the percent of total radioactivity administered that was recovered in the serum,

the percent of total radioactivity applied to the column from an aliquot of serum that was recovered in the void volume, and the intravascular volume of the animal.

Serum albumin was determined colorimetrically by the bromocresol green method (18).

Comparisons were made between groups by the student's t-test.

RESULTS

Malnourished Animals

After 4 weeks on the low protein diets, body weight, hematocrit and serum albumin were all significantly less than in the control animals. Small intestinal wet weight, DNA and protein content were also significantly less in this group (Table I).

TABLE 1

The Effect of a Low Protein Diet on Nutritional Status and on Small Intestinal Weight, DNA and Protein Content

Measured Parameter	Malnourished (N=17)	Control (N=15)	P
Body weight (g)	77.8 ± 13.6[a]	269.3 ± 30.9	<.001
Hematocrit (%)	34.3 ± 3.5	44.5 ± 2.9	<.001
Albumin (g/100 ml)	2.02 ± .28	3.65 ± .25	<.001
Intestinal weight (g)	1.92 ± .22	4.90 ± .62	<.001
Total intestinal DNA (mg)	19.5 ± 2.0	36.9 ± 5.4	<.001
Total intestinal protein (mg)	254.6 ± 31.6	603.6 ± 59.5	<.001

[a]Mean ± S.D.

Transport Studies

The total quantity of intact BSA and its large molecular weight fragments that was transported into the portal circulation from the intestinal lumen was measured.

The amount transported was expressed per g body weight to control for the difference in size between the malnourished and control groups (Figure 1). The malnourished animals showed approximately twice the uptake per g body weight as the control animals at 10, 100 and 300 mg of BSA ($p<.001$, $p<.01$ and $p<.001$, respectively).

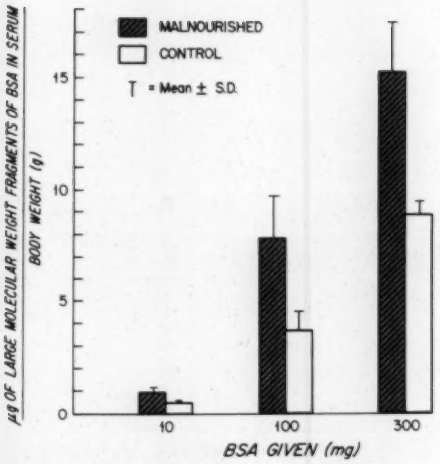

FIGURE 1

Total quantity of BSA in serum per g body weight obtained from malnourished and control rats after duodenal injection of 10, 100 and 300 mg of (^{3}H)BSA.

The amount of BSA transported was also expressed as a function of total intestinal DNA content to control for the difference in intestinal surface area between the 2 groups (Table 2). At the 10 and 100 mg doses of BSA, the malnourished animals showed greater uptake than the control animals. However, at the 300 mg dose the malnourished animals showed significantly less uptake than the control animals.

TABLE 2

Comparison of Uptake of Large Molecular Weight (LMW) Fragments of BSA per ug Gut DNA in Rats Given 10, 100 and 300 mg of BSA

	ug of LMW Fragments of BSA x 10^{-3} / ug Gut DNA		
Dose of BSA (mg)	Malnourished	Control	P
10	4.2 ± 1.0[a] (N=5)	3.6 ± 0.6 (N=5)	N.S.
100	37.1 ± 8.1 (N=6)	25.6 ± 5.6 (N=5)	<.05
300	46.5 ± 9.6 (N=6)	70.9 ± 19.8 (N=5)	<.05

[a]Mean ± S.D.

DISCUSSION

In these studies the transport of BSA from the intestinal lumen into the portal circulation was quantitated. Malnourished animals transported approximately twice the quantity of BSA as the controls per g body weight at each level of BSA given. This observation suggests that under conditions of malnutrition the intestine becomes more permeable to macromolecules. Several factors, known to occur in malnutrition, may be responsible for this phenomenon, namely decreased intestinal proteolysis due to pancreatic insufficiency (19), decreased intestinal secretory antibodies (4,5,6) and intestinal mucosal atrophy (1,2,3). Another possibility, decreased intravenous clearance of protein, will be discussed in a subsequent study (20).

The increased permeability observed in the malnourished rats was concentration dependent. Between 10 and 100 mg there was a linear increase in BSA uptake per g body weight for both groups. However, between 100 and 300 mg there was a leveling off of uptake for the malnourished animals, implying an upper limit to the absorptive capacity of this group. The same trend was observed when BSA uptake was expressed relative to intestinal DNA (Table 2). The malnourished animals showed a relatively small increase in uptake from 100 to 300 mg, 1.3-fold, compared to a 2.8-fold increase shown by the controls. This observation suggests that the mechanism responsible for macromolecular uptake in malnourished animals was saturated at a lower antigen concentration, resulting in a decreased maximum threshold for transport of macromolecules. This could be related to the decreased surface area of the malnourished gut as reflected by its lower DNA content. Another factor that could have influenced these results, by increasing the concentration gradient, was the occlusion of the lumen of the gut to prevent the loss of BSA by excretion.

Although Worthington (7) found greater absorption of intact protein in malnourished rats, this did not occur until after 2 months, unlike these studies in which a difference between groups was demonstrated at 1 month. A possible explanation for this discrepancy is that the nutritional status of the animals in the Worthington study was better, based on a greater body weight at 1 month, approximately 120 g compared to 78 g in these studies. After 2 months, the rats weighed approximately 100 g, so they were more comparable in size to the 1 month malnourished rats used here.

Although increased uptake of protein could be considered an adaptive response to a state of protein deficiency, it seems unlikely that such small amounts of protein could be of any significant nutritional benefit. A more important consideration is whether there are any harmful sequellae from increased permeability. Chandra (9) reported a higher incidence of antibodies to several food proteins in the serum of malnourished children compared to healthy ones, while at the same time noting that there were no allergic reactions when they were challenged orally with the food antigen. Olusi (21) was unable to detect food antibodies in a group of malnourished children, although he found that the immune responses in these children were so depressed that, even if food antigens crossed their intestinal mucosae, they would probably be incapable of stimulating specific antibody production. Studies are needed of rehabilitated malnourished children to determine if there are any long term effects from this such as food allergies and antigen-antibody complex disease.

These findings raise the possibility of increased intestinal permeability to intestinal pathogens and may at least partially explain the high incidence of gastrointestinal and systemic infections in malnourished children (22).

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TRANSPORT OF MACROMOLECULES IN MALNOURISHED ANIMALS
II. Intravenous Clearance After Antigen Absorption

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ABSTRACT

It has been demonstrated that increased quantities of bovine serum albumin (BSA) and/or its large molecular weight breakdown products are present in the serum of malnourished rats after its instillation into the duodenum. However, it has not been established whether this increased circulating BSA is related to enhanced intestinal permeability due to changes in the mucosal barrier or to other factors associated with malnutrition such as impaired intravenous clearance of the protein after it has crossed the mucosal barrier. To determine if altered clearance of intravascular antigens occurred, ^{125}I-BSA was injected intravenously into 4 malnourished and 4 control rats and total serum radioactivity measured both before and after precipitation with TCA at intervals for 4 hours. Uptake and degradation of ^{125}I-BSA by the liver were also measured. Both total and TCA precipitable serum radioactivity did not differ significantly between study groups at any of the times tested. Furthermore, the rate of BSA uptake by the liver was nearly identical for both groups although the malnourished degraded it to a slightly lesser extent than the controls. These findings appear to eliminate the possibility that delayed intravascular clearance accounts for increased serum levels of antigen in malnourished animals and supports the hypothesis of increased intestinal permeability to macromolecules in malnutrition.
KEY WORDS: Malnutrition, liver function

INTRODUCTION

Previous studies from this laboratory and others have shown that increased quantities of large molecular weight fragments of, as well as intact, bovine serum albumin (BSA) are present in the circulation of malnourished animals given enteric infusions of BSA (1,2). These results suggest an altered mucosal barrier in malnourished animals allowing for increased intestinal permeability to enteric antigens. However, another possible explanation for these findings is decreased intravenous clearance of the absorbed antigen, due to changes in the liver that are known to occur in malnutrition (3,4). To test this hypothesis, albumin was given intravenously to malnourished rats and blood samples collected over a period of 4 hours, the same time period used to study macromolecular transport in previous reports (1,2). The liver was examined both histologically for pathologic changes and physiologically to determine its rate of uptake of BSA and its capacity to degrade protein.

MATERIALS AND METHODS

Eight male rats of Sprague-Dawley descent (Charles River Breeding Laboratories, Wilmington, MA) were placed, at 4 weeks of age, on either a low protein (0.5% lactalbumin) or control (18% lactalbumin) diet for 4 weeks as previously described (1). At this time ^{125}I-BSA was given intravenously and blood samples collected by an aortic cannula at 5, 15 and 30 minutes and 1, 2, 3 and 4 hours. At the final time point the animals were killed by exsanguination and the liver removed. Total radioactivity in the serum and liver was measured both before and after protein precipitation with trichloroacetic acid (TCA). Liver tissue was fixed in 10% formalin, embedded in paraffin, cut to 6 micron sections, and stained with hematoxylin and eosin.

Iodinated albumin was used to study intravenous clearance, instead of tritiated albumin. This is because it has been shown that the ^{3}H-labeled material has a shorter half-life than the iodinated material (5) when given intravenously, probably due to tritium exchange in vivo. Tritiated albumin was used to study intestinal uptake of protein in Part I of this series (1) because of the findings of Parkins et. al. (6) that iodinated tracer proteins can be deiodinated by intestinal iodinases.

The albumin was iodinated by the Bolton-Hunter procedure (7). This method uses the N-hydroxysuccinamide ester of iodinated p-hydroxyphenylpropionic acid (^{125}I) for protein labeling. This radioactive ester, named Bolton-Hunter Reagent (^{125}I), reacts in an aqueous solution with primary amino groups of proteins and peptides to convert them to acyl derivatives. This acylation reaction, unlike other radioiodination procedures, acts in the absence of strong oxidants, thus minimizing the possibility of protein denaturation.

Lyophilized crystalline bovine serum albumin (Sigma Chemical Co., St. Louis, MO), 2 mg, in 200 ul of 0.1 M-borate buffer, pH 8.5, was added to 2 uCi of the iodinated ester (Bolton-Hunter Reagent ^{125}I, New England Nuclear, Boston, MA). The mixture was stirred for one hour at 0°C at which time 200 ul of 0.2 M glycine in borate buffer were added and stirred again for five minutes. Dextran 2000 was added to the mixture which was then applied to a Sephadex G-25 column (Pharmacia Co., Piscataway, NJ) in borate buffer. The void volume was collected and dialyzed in 4 liters of phosphate buffered saline (PBS) overnight. The specific activity of the protein was 4.09×10^{8} cpm/mg. This labeled albumin was used as a tracer in a stock solution of unlabeled albumin (2 mg/ml). The experiment was repeated on consecutive days, each day using two malnourished and two control rats. On the first day the specific activity of the injected material was 14.7×10^{6} cpm/mg and on the second day it was 15.1×10^{6} cpm/mg. The specific activity calculation included the protein concentration from the radioactively-labeled albumin in addition to the unlabeled albumin, bringing the total protein concentration to 1.04 mg/ml. The injected antigen was given in a total volume of 0.5 ml.

Animals were fasted overnight. A solution of supersaturated KI, 0.5 ml in 1.0 ml of PBS, was administered to all animals at the beginning of the fast by a gastric tube. Rats were anesthetized with 10% Diabutol^{R}, approximately 0.6 ml per kg of body weight, by an intraperitoneal injection. Through the midline incision the aorta was cannulated with beveled polyethylene tubing (internal diameter 0.030", Intramedic, Parsippany, NJ) as far distally from the heart as possible and close to where it bifurcated to form the femoral arteries. At the time of the aortic cannulation an aortic ligature was placed which occluded blood flow to the distal extremities. When the cannula was in place a small incision was made in the left femoral region and the saphenous vein located. Using a tuberculin syringe with a 27 gauge needle, radiolabeled albumin, 1 mg in a total volume of 0.5 ml saline (15.2×10^{6} cpm on the first day and 15.7×10^{6} cpm on the second day) was slowly injected into the vein. All animals received equal quantities of antigen. After the injection, aliquots of blood, approximately 0.2 ml, were collected at 5, 15 and 30 minutes and 1, 2, 3 and 4

hours from the aortic cannula. The animals were then killed by exsanguination and the liver, after injection with heparinized saline to clear it of blood, removed and stored at 4°C. The wet weight was recorded and total radioactivity measured.

To determine the rate of uptake of albumin by the liver, the following equation was used:

$$A = r\int_0^t c\, dt$$

where A is the radioactivity of the liver per gram (cpm/g), r is the rate of uptake of the albumin per gram of liver (ml/min x g), c is the radioactivity per ml of serum (cpm/ml), and t is the time in minutes. This equation was solved for rate:

$$r = \frac{A}{c_o} \frac{k}{1-e^{-kt}}$$

where k is the slope of the regression line of log blood level plotted against time and c_o is the initial concentration of the radioactivity in the plasma (cpm/ml) (8).

The percentage of acid insoluble radioactivity was determined in the liver which was homogenized in 10 ml of PBS (9). One ml of 20% TCA was added to 1 ml of the homogenate, iced for 20 minutes and then centrifuged for 20 minutes at 2500 rpm. The supernatant was then decanted and the pellet washed with 1 ml of 10% TCA and centrifuged as above. The radioactivity in the supernatant and pellet was measured. The percentage of acid insoluble radioactivity was calculated by dividing the radioactivity in the pellet by the total radioactivity (pellet and supernatant).

Serum samples were collected after centrifugation of whole blood to remove red blood cells. They were then counted in a gamma counter (Gamma 7000, Beckman, Fullerton, CA) before and after protein precipitation with TCA, which was done as described above, using 0.05 ml of serum.

To determine the total percentage of the radioactivity remaining in the vascular compartment, total serum volumes were determined by a dilutional technique (10). They were calculated by dividing the total amount of radioactivity given to the animal by the radioactivity in 1 ml of serum which was sampled five minutes after injection. This is considered to be sufficient time for equilibration of the isotope with the extravascular space (10).

Serum values were analyzed using nonpaired t-tests and linear regression. Tissue values were analyzed using nonpaired t-tests.

RESULTS

Malnourished Animals

After 4 weeks on the special diets, the protein-deficient rats weighed 87 ± 8.8 g and the controls weighed 322 ± 16.8 g ($p<.001$) (mean ± S.D.). Standard parameters used to define malnutrition were reported in a previous publication (1).

Clearance of Serum BSA

The log of total serum radioactivity plotted against time is shown in Figure 1. Analysis by linear regression showed no significant difference between groups in serum disappearance of the injected dose. The slopes and y-intercepts of the lines were -.001835 and 16.505 for the malnourished and -.001589 and 16.551 for the controls, respectively.

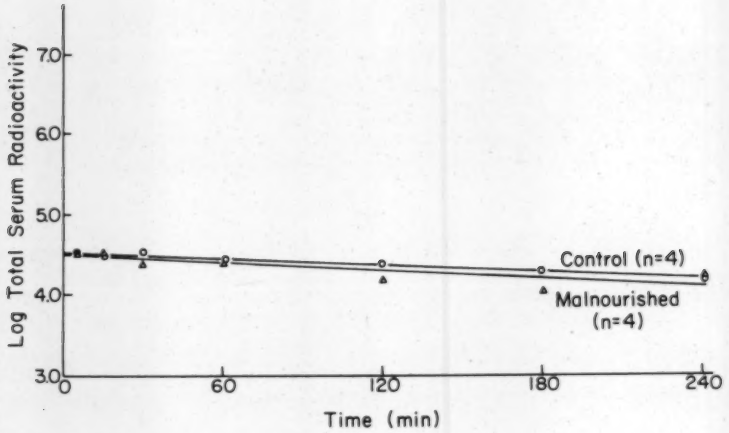

FIGURE 1

Rate of serum disappearance of ^{125}I-BSA after intravenous injection in four malnourished and four control rats.

Although there was a decrease in serum radioactivity during the 4 hour study period, the percentage that was acid insoluble did not change. As with total radioactivity, there were no significant differences between groups at any time. The mean percentage of TCA precipitable radioactivity was 91.6 (±6.1) for the malnourished animals and 95.0 (±2.5) for the control animals at 5 minutes. At 4 hours this percentage was 94.2 (±2.6) for the malnourished and 96.4 (±1.2) for the controls (mean ± S.D.).

Liver Distribution of BSA

Liver uptake and degradation of BSA is summarized in Table 1.

TABLE 1

Liver Uptake and Degradation of Radiolabeled Albumin ($\bar{x}$ ± S.D.)

	Malnourished (N=4)	Control (N=4)	P
Liver weight (g)	3.73 ± 0.83	11.50 ± 1.22	<.001
Weight as % of total body wt.	4.30 ± 0.22	3.58 ± 0.19	<.01
Uptake of BSA (ml/min x g) x 10^5	28.54 ± 3.50	32.51 ± 3.89	N.S.
Total radioactivity x 10^{-3}	620.9 ± 27.6	546.4 ± 63.2	N.S.
% acid-insoluble radioactivity	81.2 ± 3.9	69.2 ± 3.2	<.01

The liver occupied a significantly greater percentage of the total body weight in the malnourished animals than in the controls. The rate of uptake of BSA by the liver was nearly identical for both groups. However, the malnourished had a significantly higher percentage of radioactivity associated with the acid-insoluble fraction, 81.2% compared to 69.2% ($p<.01$).

Morphologic sections of the malnourished liver showed normal lobular architecture and normal Kupffer cells. There were some degenerative changes, however, compared to controls. Lobular staining was abnormal, with poor uptake of eosin. The hepatic cords were not well defined and were distended with vacuolated cells. The hepatocytes showed definite although mild, fatty change diffuse throughout the lobule. The cytoplasm was distended with numerous small to moderate size vacuoles.

DISCUSSION

The purpose of this study was to determine whether there was impaired clearance of antigen from the systemic circulation in malnutrition. Results from previous studies (1,2) suggested a defect in mucosal barrier function in malnutrition, based on finding increased quantities of BSA in the plasma 4 hours after its administration. However, these results may have been due to impaired hepatic clearance of the albumin. The Kupffer cells of the liver play a major role in removing macromolecules from the portal blood, thereby regulating the amount of antigen entering the circulation (11).

To study this, the rate of disappearance of ^{125}I-BSA after intravenous injection was measured for 4 hours in malnourished rats. The results obtained strongly suggest that liver clearance function was not impaired in these animals based on finding no significant difference between groups for both total serum and acid-insoluble radioactivity over time. The rate of albumin uptake by the liver was also similar for both groups. The only difference noted was that the malnourished appeared to degrade the albumin in the liver to a slightly lesser extent than the controls, based on finding a higher percentage of acid insoluble radioactivity in this organ. This observation may reflect a deficit of lysosomal enzyme function in the Kupffer cells as a result of malnutrition.

These findings support the conclusions of earlier studies (1,2) in which increased quantities of BSA in the circulation were attributed to an altered mucosal barrier due to malnutrition, resulting in increased intestinal permeability. Further work is needed to establish what factor or factors are responsible for this decrease in barrier function in malnutrition.

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EFFECT OF DIETARY CALCIUM DEFICIENCY ON BONE MATRIX-INDUCED ECTOPIC BONE

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ABSTRACT

The effect of dietary depletion of calcium on collagenous bone matrix-induced ectopic bone was studied, following thirty days of deficiency. While the molar ratio of phosphorus and calcium did not exhibit any significant differences between the control and experimental groups, individual concentrations of these minerals showed significant reduction in the ossicles derived from experimental rats. A concomitant decrease and increase in urinary excretion of calcium and phosphate was noted in the calcium deficient group; the serum levels of these parameters, however, were not affected. Both hexosamine and hydroxyproline levels appeared higher in calcium deficient ossicles. A significant elevation of alkaline and acid phosphatase as well as aryl sulfatase was observed in calcium deficient ossicles. There appeared to be no difference between control and experimental rats in the rate of calcification as measured by ^{45}Ca uptake. These results suggest that dietary deficiency induces significant resorption of ectopic bone without altering mineralization capacity. It is suggested that matrix-induced ectopic bone may be used as a suitable experimental model to study pathophysiology of bone loss under various experimental conditions.

KEY WORDS: Calcium deficiency, ectopic bone, ^{45}Ca uptake, lysosomal enzymes.

INTRODUCTION

Osteogenic differentiation induced by decalcified bone matrix has successfully been used as an experimental model to study endochondral bone development (1-4). Due to discrete temporal pattern of its development (3,4) it has not only been possible to identify specific biochemical changes associated with these developments but has also been advantageously exploited to study the effect of various hormonal and non-hormonal agents on these sequences (4-6). While this was used primarily as a developmental model for endochondral bone, little is known about the physiological regulation of these ossicles following the completion of their development. Such studies are of importance in view of further exploitation of these ectopic ossicles as an experimental model for various pathophysiological studies pertaining to skeletal system. Moreover, recent use of demineralized bone matrix in the repair of craniofacial abnormalities in the human (7,8) necessitates further knowledge about the

physiology and long term stability of the ectopically induced bones under both normal and adverse conditions. These considerations prompted us to undertake a study to gain further insight into physiological regulation of ectopic ossicles under different experimental situations as well as to explore their use as models for experimental pathogenesis of bone. In the present paper we wish to report the effect of dietary deficiency of calcium on the ectopically induced bones.

METHODS

Male Long-Evans rate (~100 g BW) were implanted with powdered, demineralized bone matrix in bilateral sites of anterior thoracic region as described previously (2,3). The day of matrix implantation was designated as day 0. The implants were allowed to undergo complete cycle of development, which takes about 3 weeks (3), to form hematopoietic ossicle, as confirmed by random histological sampling. Four weeks after matrix implantation the rats were divided into two groups: (i) control, fed normal high calcium diet, and (ii) rats fed calcium deficient diet and deionized water.

Normal high calcium diet was obtained from Agway Inc. was formulated according to National Academy of Science USA specifications (1978) for salt and vitamins and consisted of 0.97 percent calcium and 0.85 percent phosphorus. The calcium deficient diet, prepared according to Kenny and Munson (9) and obtained from United Biochemical Corporation, contained 0.013 percent calcium whereas the phosphorus level was brought up to 0.85 percent by addition of KH_2PO_4. The diets and water were provided ad libitum.

In a representative experiment following 3 weeks of dietary restriction of calcium the rats were placed individually in metabolic cages supplied with water, starved overnight for 12 hours and urine was collected over the next 24 hours. The urine was centrifuged at 4,000 xg for 10 min and the aliquots of supernatant were used for biochemical analysis. In all experiments the rats were autopsied 30 days following dietary restrictions at which time biochemical analyses and histological examination of the induced ossicles were performed. At autopsy the ossicles were freed from adhering tissue, weighed and processed as per experimental schedule. In some experiments prior to autopsy a sample of blood was withdrawn by cardiac puncture and centrifuged for the analysis of serum levels of calcium and inorganic phosphorus.

Biochemical Analysis of Induced Bone

Individual ossicles derived from control and calcium deficient rats were weighed and homogenized in a buffer containing 0.15 M NaCl, 50 mM Tris HCl and 0.1 percent Triton X-100 at pH 7.2, centrifuged at 10,000 xg in a refrigerated centrifuge. The supernatants were used for analyses of alkaline phosphatase and acid phosphatase and aryl sulfatase activities.

Alkaline phosphatase, acid phosphatase and aryl sulfatase were determined in a buffer extract of the ossicles using hydrolysis of p-nitrophenyl phosphate at pH 9.3 and 5.5 and p-nitrocatechol sulfate at pH 5.5 respectively (2,10). The results were expressed as micromoles (U) of substrate hydrolysed at 37°C at 30 min.

In another experiment the ossicles were weighed and placed in 6N HCl, hydrolyzed *in vacuo* for 24 hours at 110°C. The hydrolysates were evaporated at 60°C in a Buchler rotary evaporator under vacuum, reconstituted with distilled water, and centrifuged to settle the insoluble debris. Calcium

levels in the supernatant were determined in a Perkin-Elmer atomic absorption spectrometer according to their bulletin No. 303-0152. Inorganic phosphate was determined according to Chen et. al (11). Hydroxyproline and hexosamine levels were determined according to methods described by Rojkind and Gonzalez (12) and Gatt and Berman, respectively (13).

Analysis of ^{45}Calcium Uptake

Mineralization of ectopic bone was assessed essentially according to Anderson and Sajdera (14), by following the *in vitro* uptake of ^{45}Ca by the ossicles as follows: at autopsy the ossicles were cut into small segments of 20-25 mg and 5-6 wedges were placed into 10 ml of calcifying buffer at pH 7.4 containing 85 mM NaCl, 15 mM KCl, 10 mM NaH_2CO_3, 50 mM Tris, 1.6 mM KH_2PO_4 and 2.2 mM $CaCl_2$ and preincubated in a shaking water bath at 37°C. The buffers were aerated with 95 percent oxygen and 5 percent CO_2 mixture prior to incubation. Following 30 min of preincubation 20 µci of $^{45}CaCl_2$ (sp. act. 31.8 mCi/mg, ICN) in 100 µl of buffer was added to the medium and the incubation was continued for two hours. The radioactive medium was then replaced with fresh medium and post incubation was carried out for additional 30 min. At the end of incubation, the wedges were transferred to new containers and washed with an excess volume of buffers by constant stirring with magnetic bars with 2 changes during one hour. The wedges were blot dried, weighed and placed in 6N HCl in screw capped tubes and hydrolyzed at 110°C overnight. The hydrolysates were centrifuged and aliquots of the supernatant counted for radioactivity with a Packard Tricarb Scintillation Counter. Results were expressed as cpm per mg tissue.

Calcium and phosphorus levels of urine and serum were determined following appropriate dilution according to procedures described above. Creatinine in the urine was determined according to Sigma Bulletin No. 555. The calcium and phosphate levels of urine were expressed per mg of creatine.

The biochemical results were statistically evaluated using Student's *t* test.

Histological Analysis

Regular histological analyses were done on random tissue samples using toluidine blue and hematoxylin-eosin stain.

RESULTS

Random histological evaluation to determine the developmental phase of the induced bone after one month, invariably showed hematopoietic ossicles. Calcium restriction significantly reduced urinary levels of calcium and elevated phosphate excretion. The serum levels of calcium and inorganic phosphate did not, however, show any differences. There were no significant differences in the mean body weight between the control and calcium restricted groups (Table 1).

Both calcium and phosphorus concentration in the ectopic ossicle exhibited an approximately 50 percent reduction in the calcium restricted groups, whereas no significant change in the P:Ca molar ratio was observed (Table 2). However, the concentrations of hydroxyproline and hexosamine appeared to be higher in the calcium restricted groups (Table 2). The levels of alkaline phosphatase, acid phosphatase, and aryl sulfatase activity of the ossicle also exhibited a significant (approximately fourfold) increase in the calcium deficient groups.

There was no difference in the ^{45}Ca uptake between ossicles derived from control and calcium deficient animals (Table 3).

TABLE 1

Effect of Dietary Deprivation of Calcium
on Body Weight Urinary and Serum Levels of Calcium and Phosphorus

Treatment	Body Weight (g)	Urine		Serum	
		µg Ca/mg creatinine	µg Phosphorus /mg creatinine	Calcium mg/100 ml	Phosphorus mg/100 ml
Control	385±22 (8)	44.48±4.32	140.12±13.62	11.11±0.62	6.57±0.24
Calcium Deficient	353±31 (8)	23.71±3.40[a]	457.73±37.47[b]	10.02±0.49	7.14±0.25

Number in parentheses indicates number of rats used in each study
Difference between a and b is significant $p < 0.005$
Results are shown as mean ± SEM

TABLE 2

Effect of Dietary Calcium Deficiency on Constituents of Ectopic Ossicle

Treatment	Calcium µmoles/mg	Phosphorus µmoles/mg	P:Ca*	Hydroxyproline µg/mg	Hexosamine µg/mg
Control	3.80±0.48	2.71±0.11	1:1.40	0.21±0.01	19.84±0.72
Calcium Deficient	1.76±0.07	1.39±0.06	1:1.27	0.32±0.04[a]	29.16±2.13[b]

Twelve ossicles were derived from six rats in each group
*P:Ca molar ratios in normal hydroxyapatite is 1:1.67
Results are shown as mean ± SEM

TABLE 3

Influence of Dietary Calcium Deficiency on ^{45}Ca Uptake and the Enzyme Levels of Ectopic Ossicles

Treatment	cmp/mg	Alk. Phosphatase U/G	Acid Phosphatase U/G	Aryl Sulfatase U/G
Control	17,525±1,090	13.01±0.23	27.74± 6.72	83.15± 7.11
Calcium Deficient	18,239±1,493	56.97±5.21	72.88±12.89	334.91±33.60

Eight ossicles derived from four rats in each group were utilized. Results are shown as mean ± SEM

Histological evaluation of ossicles from the calcium deficient groups revealed loss of some integrity of matrix component with occasional fibrous matrix and multinucleated osteoclast-like cells (Figs. 1 and 2).

DISCUSSION

Several studies have shown that chronic calcium deficiency induces osteoporotic changes in the bone which could be reversed by following calcium supplementation (15-18). Nevertheless, the relationship between calcium deficiency and osteoporosis is controversial (19). However, in view of reversal of calcium deficiency osteoporosis following calcium supplementation, it would appear that osteoblastic activity may not be impaired during short term deficiency, although some investigators have observed a decrease in both bone formation and mineralization (20).

The present study indicates that dietary calcium deficiency significantly reduces total calcium and phosphorus levels of the ossicles without effecting their molar ratios. While there is a definite decrease in urinary levels of calcium with a concurrent increase in urinary phosphorus, the serum levels of these parameters remained unchanged in the calcium restricted rats. Moreover, the rate of mineralization as measured by ^{45}Ca uptake remained unaffected. Thus it is highly unlikely that these conditions may be related to osteomalacia rather it appears that nutritional deficiency of calcium has resulted in the resorption of bone in the ectopic ossicles which may be a normal physiological response to restore calcium balance.

It is generally agreed that under sustained calcium deprivation an elevated level of PTH maintains calcium homeostasis at the expense of matrix minerals (20-23). However, the detailed mechanisms of action of PTH in matrix degradation is not clear, although the activation of several lysosomal enzymes have been implicated in this process (24-26). In the present study we found a significant elevation of both alkaline and acid phosphatase as well as of aryl sulfatase. A preferential loss of bone mineral, may contribute to an apparent increase in enzyme activities as well as in hydroxyproline and hexosamine levels as seen in the present study. Nevertheless, a three to four fold increase in the activities of all three enzymes concurrent to an approximately

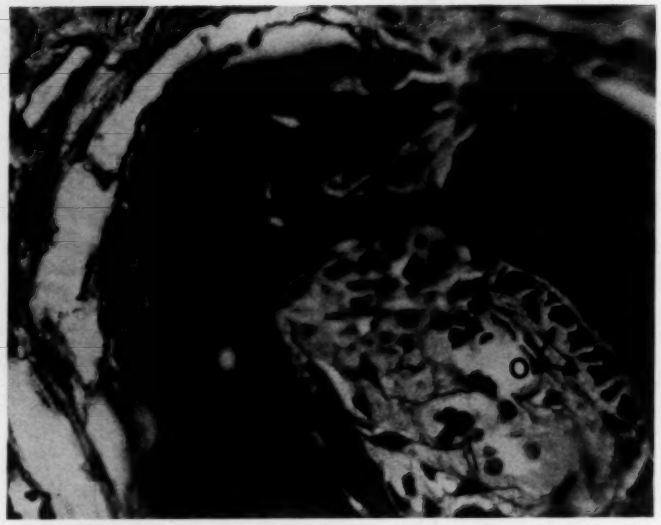

FIG. 1. Histology of ossicle derived from control rats (x 250).
Ob - osteoblast M - matrix

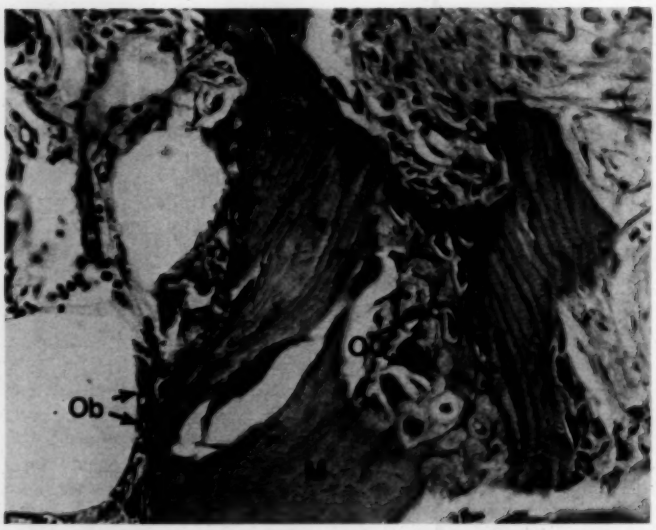

FIG. 2. Histology of ossicles derived from calcium deficient rat (x 120).
Ob - osteoblast Oc - osteoclast M - matrix

fifty percent decrease in mineral components of the ossicles in calcium restricted groups may, therefore, be significant and not due to a methodological factor. Hence an increased level of these enzymes during calcium deficiency may be responsible for the resorption of bone. Some studies have shown that PTH which induces bone resorption may stimulate alkaline phosphatase activity in bone (27,28). Since we find no evidence of concurrent increase in mineralization in the present study, it is not clear whether alkaline phosphatase, which has been traditionally associated with bone mineralization (29), may also be involved in bone resorption. However, the increase in acid phosphatase in bone resportion is in agreement with the observation of other investigators (26). We also found an increased level of aryl sulfatase, a lysosomal enzyme involved in proteoglycan degradation (10,30), in calcium deficient ossicles. Studies by other investigators have shown that protein polysaccharide degradation also take place at the site of mineralization (31). However, our earlier studies on the development of ectopic bone suggests that this enzyme is involved in the degradation of matrix by osteoclasts and macrophages during chrondrolysis and bone remodelling (10). Thus it appears that calcium deficiency induced bone resorption may stimulate degradation of proteoglycans, which may somehow be involved in the protection of the integrity of mineralized matrix.

In conclusion, our study indicated that nutritional deficiency of calcium induces resorption of ectopic bone without impairing its mineralization capabilities. We suggest that the ectopically induced bones may be used as experimental models for studying different pathological processes involving the mineralization of bone.

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ABSORPTION AND RETENTION OF $^{75}SeO_3$ IN RELATION TO SOYBEAN PROTEIN INTAKE IN THE RAT[1]

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ABSTRACT

The absorption and retention of ^{75}Se, given as an oral dose of $^{75}SeO_3$, was studied in young male rats receiving different levels and sources of soy protein, with and without selenite and methionine supplementation. Rats fed a protein-free diet had a higher cumulative urine ^{75}Se excretion and a sligtly lower ^{75}Se absorption and ^{75}Se retention than rats fed diets containing 10% protein supplied as soya flour. Results indicated that supplementation with selenite decreased the fractional absorption and retention of selenium, but the overall effect was a marked increase in the total amount of selenium ingested, absorbed and retained. Methionine supplementation of a diet based on soya increased growth and PER: it also decreased slightly cumulative feces ^{75}Se excretion and increased ^{75}Se absorption, but only in rats fed diets supplemented with selenium. The present findings are consistent with the view that selenium homeostasis in the rat is maintained largely through changes in the urinary excretion of selenium and they show that an inadequate protein diet reduces the efficiency of retention of absorbed selenite.

KEY WORDS: Selenium, bioavailability, soybean protein.

INTRODUCTION

Several factors affect the bioavailability of dietary selenium, including the level and source of selenium in the diet. For example, in chicks, the efficiency of selenium reaches a maximum at a dietary intake of 0.1 ppm and at higher levels, the percentage of intake retained declines (1). In addition, selenium bioavailability depends upon the chemical form of the element. Cantor et al. (2) reported selenium bioavailability values for plant products to range from 65% to 90% and from 9% to 25% for products of animal origin when fed to chicks. In both animal and vegetable products selenium is present as a component of proteins, but in plant products it also exists as seleno amino acids, such as selenomethionine and selenocystine. For chicks, selenium from selenite is more available than from selenomethionine or from other organic or inorganic forms. In rats, selenium from selenomethionine may be somewhat more available than from selenocystine (3), selenite (4), fish muscle (5) and rabbit kidney (6).

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In man, selenium from selenomethionine was shown to be more available than from fish muscle (7) and selenite (8, 9).

Recently Lane et al. (10) indicated that different protein sources may be of importance in determining the absorption of selenium from the rat alimentary tract. Furthermore, Gabrielsen and Opstvedt (11) showed that selenium in soy bean meal is less available than that in fish meal and corn gluten meal. Hence the present study was initiated on the basis of these observations and we chose to study in rats the possible effects of factors associated with the nutritional value of soybean proteins on the absorption and retention of a supplement of selenium, using $^{75}SeO_3^{-2}$ to trace the fate of selenium.

MATERIALS AND METHODS

Experimental design

Four groups, each consisting of six Sprague-Dawley (Charles River Breeding Laboratories, Wilmington, MA) male rats, weighing initially 64±5 g, comprised experiment I. For experiment II the design was similar, with mean initial weight of each group being 62±5 g. The rats were placed individually in metabolic cages. They were fed the experimental diets ad libitum for three days and then at the beginning of the fourth day, they received an oral dose of 1 µCi ^{75}Se-selenite. The rats were continued on the experimental diets for a further seven days. Urine and feces were collected daily from each rat, and stored in polyethylene vials at -10°C until analyzed. Daily feed intake was measured for each rat by collecting and weighing the remaining allotment of food given at the beginning of each 24-hour period. Weight gain was measured for each rat between the first and the tenth day of the experiment. A "protein efficiency ratio" (g gain per g protein consumed) was measured for each diet group.

Diets

The diets were prepared according to Campbell's (12) formulation. The basal diet contained, on a dry basis, 80% corn starch, 10% corn oil, 5% non-nutritive cellulose, 4% Hegsted (13) salt mixture and 1% Rogers and Harper (14) vitamin mixture. Except for diet 1 of experiment I, all diets contained 10% protein (N x 6.25), added at the expense of corn starch. The diets were prepared by mixing equal amounts of dry diets and 4% agar solution in distilled water, as described by Rogers and Harper (14). The raw soya flour was obtained from A.E. Staley Manufacturing Co., Decatur, IL (Extracted Soy Flour I-200), and the soy isolate (Supro-620) from Ralston Purina Company, St. Louis, MO. The soya flour used in diets 3 and 4 was autoclaved for 30 min, using a procedure described by Borchers et al. (15) to reduce trypsin inhibitor activity. Supplemental selenium, incorporated into diets 4 (1 ppm), 6 (0.25 ppm) and 8 (0.25 ppm) was added as sodium selenite. All the other diets contained less than 0.07 ppm Selenium as measured by Neutron Activation Analysis.

Urine and feces analysis

Each complete daily urine sample was diluted to 20 ml with distilled water. Fecal samples were placed in a 250 ml beaker and 40 ml concentrated HNO_3 added; the beaker, covered with a watch glass, was then placed on a hot plate and the solution brought to boiling for 20 min in order to reduce volume to about 5 ml. After cooling, the contents were transferred into polyethylene vials and diluted to 20 ml with distilled water. Samples of the (^{75}Se) selenite solution administered to the rats were also placed in 20 ml polyethylene vials and diluted to 20 ml with distilled water. The radioactivity in the urine, feces and (^{75}Se) standard solutions was monitored using the 265 kev peak of ^{75}Se (Canberra Multichannel Analysis System 8180, Canberra Industries, Inc., Meriden, CT). Results for one week cumulative excretion of ^{75}Se in urine and

feces were expressed as percent of administered dose. Selenium retention was determined by the difference between the cumulative fecal and urinary excretion of radioactivity and administered dose during the week following administration of the isotope.

^{75}Se absorption was estimated by the method of Lutwak (16) as described for selenium-75 by Thomson and Stewart (4). Cumulative daily excretion of ^{75}Se in feces was plotted against time and the line joining the points for the last four days of the experiment was extrapolated back to the zero-time intercept in order to determine the fraction of administered ^{75}Se that was not absorbed.

Statistical procedures

All values obtained in each experiment for weight gain, food intake, protein efficiency ratio, cumulative urine ^{75}Se excretion, cumulative feces ^{75}Se excretion, ^{75}Se retention and ^{75}Se absorption were subjected to analysis of variance, as described by Snedecor and Cochran (17). The degree of significance of simple orthogonal comparisons was assessed between treatments.

RESULTS

The results for the first experiment (Exp. I) are given in Table 1. Rats fed the protein-free diet showed a weight loss of 11.9 g compared to a weight gain of 9.1 g achieved by the group fed the raw soya flour diet. As anticipated, rats fed the heated soya flour diets showed a significantly ($P<0.05$) higher protein intake and about double the weight gain compared with rats fed the raw soya flour diet; consequently, the heated soya flour diets showed a higher PER than that of the raw soya flour. Selenium supplementation of these diets had no significant effect on protein intake, body weight gain or PER values.

Compared to groups 2 and 3 fed the soya flour protein diets, the group fed the protein-free diet had a high ($P<0.05$) cumulative urine excretion of ^{75}Se (15% and 28% respectively); the ^{75}Se retention was 57% for the 0% protein group compared to 71% and 73% for the 10% protein groups; ^{75}Se absorption was also slightly higher for group 2 ($P<0.075$) compared to the group fed the 0% protein diet.

The unheated and heated soya flour groups showed no significant differences in the pattern or total output of urinary and faecal ^{75}Se, ^{75}Se retention and ^{75}Se absorption.

The selenium supplemented group showed a cumulative urine ^{75}Se excretion of 51% of the dose after one week compared to 15% for the unsupplemented groups; cumulative excretion of ^{75}Se in feces was 23% compared to 12%, ^{75}Se retention was 26% compared to 73% and ^{75}Se absorption was 84% compared to 95%; all these comparisons were significantly different ($P<0.01$).

The results for experiment II are summarized in Table 2. Comparisons made between groups 5 and 6 or 7 and 8 revealed no significant effect of selenium supplementation on protein intake, rat growth or PER values. However, the cumulative urinary excretion of ^{75}Se increased from 9% and 10% of the administered dose for rats fed the unsupplemented diets to 39% and 41% for the rats fed the selenium supplemented diets. For feces, cumulative ^{75}Se excretion was raised from 10% and 11% to 15% and 17%; ^{75}Se retention and ^{75}Se absorption decreased from 80% to 44% and 96% to 90% respectively, as a consequence of selenium supplementation. Groups 5 and 6 fed the soy isolate diets achieved weight gains of 45 and 39 g compared to 63 and 71 g for groups 7 and 8 fed the methionine supplemented soya isolate diet. Thus, PER values were also significantly increased, from 3.4 and 3.3 to 4.5 and 4.6, following methionine

TABLE I

Experiment I

Weight gain, protein intake, protein efficiency ratio, urine and feces ^{75}Se excretion, ^{75}Se retention and absorption measured in rats.

Diet Treatment No	Identification	Weight gain g/10 days	Protein Intake g/10 days	Protein Efficiency Ratio	Cumulative Urine ^{75}Se Excretion[1] (A)	Cumulative Feces ^{75}Se Excretion[1] (B)	^{75}Se Retention 100-(A+B)[1]	^{75}Se Absorption (% of dose)[1]
1	0% protein	-11.9[2] ±0.9	-	-	28.0 ±8.4	14.6e ±2.3	57.4 ±6.9	92.3h* ±1.9
2	Raw soya flour	9.1 ±5.3	6.7 ±1.0	1.30 ±0.67	15.1d ±4.5	13.8ef ±1.3	71.3g ±4.2	94.4hi* ±1.2
3	Heated soya flour	20.8a ±5.1	8.8b ±0.8	2.32c ±0.36	14.7d ±4.7	12.4f ±1.3	72.8f ±4.1	94.9i ±0.9
4	Heated soya flour +1.0 ppm Se	18.6a ±5.7	8.4b ±0.7	2.20c ±0.52	50.9 ±7.4	22.9 ±1.9	26.0 ±8.1	84.4 ±2.2

[1] Feces and urine ^{75}Se excretion, ^{75}Se retention and ^{75}Se absorption are expressed as percentage of the dose given.

[2] Mean ± SE for 6 rats per group.

* Mean values significantly different for $P<0.075$.

[a-i] Mean values with the same superscript letter were not significantly different ($P<0.05$).

TABLE 2

Experiment II

Weight gain, protein intake, protein efficiency ratio, urine and feces ^{75}Se excretion, ^{75}Se retention and absorption measured in rats.

Treatment No	Identification	Weight gain g/10 days	Protein Intake g/10 days	Protein Efficiency Ratio	Cumulative urine ^{75}Se Excretion[1] (A)	Cumulative Feces ^{75}Se Excretion[1] (B)	^{75}Se Retention 100-(A+B)[1]	^{75}Se Absorption (% of dose)[1]
5	Soya isolate	44.6a ±10.3	13.1cd ±1.4	3.37f ±0.46	9.62h ±2.7	10.6j ±0.7	79.8ℓ ±2.8	95.7n ±0.4
6	Soya isolate +0.25 ppm Se	39.3a ±8.4	11.8c ±1.6	3.29f ±0.31	38.9i ±7.0	17.0 ±1.1	44.1k ±7.7	88.9m* ±0.6
7	Soya isolate +0.1% L-Meth.	62.8b ±11.2	14.0de ±2.0	4.47g ±0.16	10.3h ±2.4	9.8j ±1.0	79.9ℓ ±1.9	95.9n ±0.7
8	Soya isolate +0.25 ppm Se +0.1% L-Meth.	71.4b ±4.4	15.5e ±0.7	4.62g ±0.20	40.8i ±5.3	15.2 ±2.1	43.9k ±4.2	90.1m* ±1.7

[1] Feces and urine ^{75}Se excretion, ^{75}Se retention and ^{75}Se absorption are expressed as percentage of the dose given.

[2] Mean ± SE for 6 rats per group.

* Mean values significantly different for $P<0.075$.

a-n Mean values with the same superscript letter were not significantly different ($P<0.05$).

supplementation. There was a significant but small decrease in the cumulative fecal excretion of ^{75}Se ($P<0.075$) when soy isolate was supplemented with methionine (groups 6 and 8).

DISCUSSION

The results of experiment I indicate that although the rats fed the 0% protein diet absorbed 92% of the dose of $^{75}SeO_3$, compared to 94% and 95% for the rats fed the 10% protein diets, they also excreted 28% of the administered dose via the urine during the following week compared to only 15% for the rats fed the diet containing protein. This shows that the level of dietary protein has only a small statistically significant effect on the absorption of selenium as selenite but it has a greater effect on the retention of absorbed selenium. The latter increases therefore, with a reduced level of dietary protein, at least in comparing a protein-free diet with a diet supplying 10% protein. In contrast to our findings, Tougas et al. (18) reported that a low protein and high phosphorous diet produced higher fecal excretion in adult man compared to a high protein, low phosphorous diet. Said and Hegsted (19) showed, however, that different levels of dietary protein had no significant effect on the excretion of absorbed selenium in the rat when ingested as ^{75}Se-selenomethionine.

The results for selenium supplementation were consistent in both experiments; no significant differences were observed for food intake, weight gain and PER, and the findings indicated a lower fractional absorption and retention of ^{75}Se for rats fed the selenium supplemented diets. However, when the results for absorption and retention were expressed in absolute amounts of selenium, as shown in Table 3, comparisons between groups 3 and 4 show that the rats fed the selenium-supplemented diet ingested 81 times more selenium, absorbed 72 times more selenium and retained 29 times more selenium than the rats fed the unsupplemented diets. Similarly for experiment 2, supplementation of diets with selenium resulted in a 15- to 18-fold increase in selenium ingestion; absorption was increased by 13- to 17-fold and retention by 8- to 10-fold. Methionine supplementation did not affect the response of selenium metabolism to dietary selenium supplementation except by an influence on total food intake.

TABLE 3

Total amounts, (μm) per 10 days, of selenium ingested, absorbed and retained.

Experiment I Group	Diet Identification	Se ingested	Se absorbed	Se retained
1	0% protein	0.16	0.15	0.09
2	raw soya flour	0.84	0.79	0.59
3	heated soya flour	1.05	0.99	0.76
4	heated soya flour + 1 ppm Se	84.70	71.51	22.00
Experiment II				
5	soya isolate	2.13	2.04	1.70
6	soya isolate + 0.25 ppm Se	31.43	27.93	13.81
7	soya isolate + 0.1% L. Meth.	2.26	2.17	1.81
8	soya isolate + 0.25 ppm Se + 0.1% L. Meth.	41.24	37.16	18.12

The effect of methionine supplementation was measured without selenium supplementation (comparison between groups 5 and 7) or with selenium supplementation (comparison between groups 6 and 8). In both cases, as expected, the weight gain and PER were each increased significantly by methionine supplementation of the soya isolate diets. Only the comparison between groups 6 and 8, both supplemented with 0.25 ppm selenium, showed a lower cumulative feces ^{75}Se excretion ($P<0.05$) and a higher ^{75}Se absorption ($P<0.075$) by the group fed the methionine supplemented diet. According to Said and Hegsted (19) and Ewan et al. (20), methionine had no effect on the excretion of selenium in the rat. Sunde et al. (21) showed that a low intake of dietary methionine decreased the biopotency of selenomethionine in the rat, as evaluated by a glutathione peroxidase test, but had no effect on the biopotency of selenite. Hansson (22) found that pre-treatment of mice with methionine reduced the incorporation of selenium into pancreatic juice implying a possible competition for absorption between methionine and selenomethionine. In the present experiment, methionine supplementation did not alter the utilization of selenium when given as ^{75}Se selenite, except through a change in total food intake and thus, in the absolute selenium ingested and retained.

Based on the combined results of the two experiments, it may be concluded that the adequacy of the protein intake level influences the retention of absorbed selenium but the type and quality of soy protein does not have any important influence on the overall metabolism of dietary selenium, at least when the source is ^{75}Se-selenite. Heat treatment of soy flour had a positive effect on food intake, weight gain and PER, but none of these effects were correlated with availability of ^{75}Se when given as selenite. This observation indicates that the heat treatment had a beneficial effect on the nutritional quality of the soya flour protein, presumably due to increased digestibility of the soy protein. Thus, as shown by Cantor et al. (2) in chicks, and from our results, it appears that the availability of selenite in rats is not dependent on the digestibility of the soy and that the presence of active trypsin inhibitors does not affect bioavailability of this form of selenium.

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PHOTOGONADAL RESPONSES IN VITAMIN A DEFICIENT COCKERELS[1,2]

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ABSTRACT

Two experiments were conducted to determine if the progonadal effect of a moderate hypovitaminosis A state in cockerels was due to alterations in photoreception either via the eyes or extraretinal photoreceptors. In experiment one, day-old Single Comb White Leghorn cockerels were fed either a vitamin A adequate or marginally deficient ration and subjected to one of three lighting regimes (10:14, 14:10 or 24:00 hr of light:dark). Mildly A-deficient chicks exhibited lighter body weights than controls. In contrast, comb and testes weights (expressed as percentages of body weights) became greater for deficient birds compared to controls as they aged. This trend was noted in all lighting regimes. Lighting durations of 24 and 14 hours (averaged over diet) produced greater percent comb and percent testes weights than 10 hours. These data indicate that a mild vitamin A deficiency produces a progonadal effect despite non-stimulatory lighting. In experiment two, blinded cockerels were fed either the control or marginally deficient diet and subjected to one of four light intensities (.24, 6.00, 12.08 or 37.53 lux). Body weights were again less in mildly A-deficient birds as compared to controls. Within A-deficient treatments, percent comb weights of the 6.00 lux chicks were greater than those in 37.53 lux. No differences were noted among the remaining treatments in percent comb weight. A significant interaction between the dietary vitamin A level and light intensity on percent testes weight was found. Marginally A-deficient cockerels had greater percent testes weights than corresponding controls at 6.00 and 12.08 lux. These data indicate that a mild vitamin A deficiency alters photogonadal response via extraretinal photoreceptors.

KEY WORDS: Vitamin A, cockerels, photogonadal, light intensity, extraretinal

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INTRODUCTION

The fundamental physiological functions of vitamin A, besides its involvement in the visual cycle and now possibly glycoprotein synthesis (1) remain nebulous. The vitamin is known to be essential for life, growth and reproduction (2) but the means by which it influences these processes has not been elucidated. In reproduction, a severe deficiency of this vitamin has been demonstrated to produce inhibition of testicular maturation in California quail (3), decreased semen quality in adult roosters (4) and gonadal atrophy and/or retardation in male rats (5-7). In contrast, mildly avitaminotic A growing cockerels have exhibited increased testes and comb weights and advanced gonadal development (8-11).

The presence of light is known to be stimulatory upon pituitary secretion of luteinizing hormone and follicle-stimulating hormone (12, 13) while increasing day length results in greater pituitary prolactin concentrations in chickens (14) and Japanese quail (15). As a result of diurnal variations in these pituitary hormones, subsequent increases or decreases in sexual capacity ensue. Long photoperiods (greater than 12 hours of light per day) have been shown to have a stimulatory effect upon sexual fecundity and maturation of many avian species, while short days (less than 12 hours of light per day) are generally nonstimulatory (16-20).

Vitamin A is absolutely necessary for light perception via the retina. However, eyesight is not essential for a photosexual response to occur in many avian species, though under certain conditions retinal light perception does modify this effect (21-25). Despite confirmation of their existence, the precise location(s) of these nonretinal photoreceptors remains elusive (26).

The present experiments were undertaken to determine if a mild vitamin A deficiency alters photoreception so that the onset of sexual maturation is markedly advanced and if so, whether extraretinal photoreceptors are involved.

METHODS

Experiment One: A 2x2x3 factorial design, consisting of two levels of vitamin A (control and marginally deficient), three lighting regimes (24:00, 14:10 and 10:14 hr of light:dark), and three ages for data collection (8, 10 and 12 weeks of age) were utilized.

Two hundred seventy day-old Single Comb White Leghorn (SCWL) cockerels were weighed, wing banded and randomly allotted to one of three environmentally light and temperature controlled rooms and to one of two diets within each room. The control and marginally vitamin A deficient rations were prepared by adding either 6,600 or 660 IU retinyl palmitate per kg of basal ration, respectively (Table 1). The chicks were reared in raised, wire-floor cages until five weeks

TABLE 1

Composition of Basal Diet.

Ingredient	Percent
Ground milo	63.73
Soybean oil meal, solvent process (44%)	32.40
Dicalcium phosphate	1.90
Limestone	1.15
Salt, iodized	0.50
Methionine, hydroxyanalog	0.22
Mineral premix[a]	0.09
Vitamin premix[b]	0.01

[a]Mineral premix nutrients per kilogram diet: manganese sulfate, 90 mg; ferrous sulfate, 90 mg; zinc sulfate, 90 mg; calcium carbonate, max. 45 mg, min. 36 mg; copper oxide, 9 mg; potassium iodide, 2.7 mg; cobalt carbonate, .9 mg.
[b]Vitamin premix nutrients per kilogram diet: vitamin D_3, 1101 IU; ∝-tocopheryl acetate, 40 IU; choline, 1980 mg; Ca-pantothenate, 2.63 mg; riboflavin, 2.54 mg; vitamin K, 2.20 mg; niacin, 1.70 mg; cyanocobalamin, 11.01 µg.

of age when they were transferred to growing batteries. Room temperature was regulated to provide standard growing conditions. Fifty chicks were allocated to each vitamin A deficient group, while 40 were assigned to each control group. Each room was lighted by a single, 100-watt, incandescent bulb situated at the center of the ceiling. Continuous lighting (24:00 hr) was maintained until the chicks reached two weeks of age, at which time the three differential lighting schedules were imposed. Feed and water were provided ad libitum.

Beginning at 0800 hour on weeks 8, 10 and 12, six chicks from each treatment were randomly selected, weighed and sacrificed by decapitation. Comb and testes were removed, weighed and recorded.

Experiment Two: Seventy-five day-old SCWL cockerels were randomly allotted to either a control vitamin A adequate or marginally vitamin A deficient diet. To the same basal diet (Table 1), 6,600 or 660 IU retinyl palmitate was added per kg for control or A-deficient diets. The chicks were housed in temperature controlled raised wire-floor batteries. Feed and water were provided ad libitum and a lighting schedule of 12:12 hours (light:dark) was maintained. Light was provided by standard fluorescent bulbs. Chicks anesthetized with chloroform had unilateral ocular enucleations performed when 10 days of age. After severing the surrounding musculature and optic nerve, a small cotton ball was placed in the socket and the eyelid sutured closed. One to three days of recovery followed, after which the remaining eye was removed.

At five weeks of age 20 chicks from each diet demonstrating positive weight gains and not exhibiting severe eye infections were randomly allocated to one of four light intensities (.24, 6.00, 12.08 and 37.53 lux). Three chicks per treatment were housed individually, while the other two were caged together as replacements. Light was provided by broad-spectrum fluorescent bulbs mounted three feet from the center of each row of cages. Intensity was varied by multiple layers of fiberglass screening and measured at chick head-height on a Luna-Pro System Exposure Meter (Photo-Tech Inc., New York, NY) (27).

Following a two-week adjustment period, the activities of the singly housed chicks were monitored on an event recorder to ascertain if the birds could perceive the prevailing photoperiod. This was achieved by attaching a length of parcel string to the bird's leg, guiding it through a small hole in the rear of the cage, over a pulley and weighting it with lead. Movement of the pulley tripped a microswitch (Newark Electronics, Denver, CO; model V3L-111D8) which triggered an Esterline Angus event recorder (27). To assure that the chicks were not cueing on a particular event, they were inspected, fed and watered at a different time each day after the start of the light phase. No attempt was made to prevent vocal communication.

Serial selection of chicks was practiced to eliminate possible diurnal effects upon sampling. Birds were sacrificed by decapitation at 15 weeks of age. Body, liver, testes and comb weights were recorded. Blood was collected from the jugular vein in heparinized centrifuge tubes. The plasma samples were stored at -4°C for vitamin A analysis via cyclohexane extraction using the method of Kahan (28). Livers were placed in sample bags, frozen in liquid nitrogen and stored at -4°C for vitamin A analysis (28).

Data for both experiments were analyzed using a two-way factorial analysis of variance (29). Mean separations were performed utilizing Duncan's multiple-range test (30).

RESULTS AND DISCUSSION

Chicks ingesting the marginally vitamin A-deficient diet weighed significantly less ($P<.05$) than controls at 8 and 10 weeks of age but not at 12 weeks in experiment one (Table 2). Although a depression in body weight due to a mild A-deficiency was noted, the chicks continued to grow at a rate that paralleled controls (Table 2). These findings support those reported by Nockels and Kienholz (8) and Nockels et al (31). In contrast, Nockels and Herrick (9) found no influence of a marginal A-deficiency on body weight.

Photoperiod duration (averaged over diet) also influenced body weight (Table 2). Chicks given 10 hours of light were heavier ($P<.05$) than those receiving 24 hr, while birds of the 14 hr treatment were intermediate at all ages examined. These data are in opposition to those of Weaver and Siegel (32) who stated that broiler cockerels maintained in constant light were heavier at 56 days of age than those in 8:16 hr of high intensity to low intensity or high intensity to darkness. Though their data did not prove to be significant, Squibb and Collier (33) noted a trend similar to the previous study. Possibly, there is a critical time span between 10 and 8 hours of light required for chicks to consume an adequate quantity of feed to compensate for the extended dark period. Those in constant darkness appear to be able to adapt as shown by the work of Shirley and Osei (34), who found that constant light or darkness caused significantly superior body weights in Hyline W-36 (Leghorn) chicks over those in a diurnal regime (12:12 hr).

TABLE 2

Affects of Dietary Vitamin A, Lighting Regime and Age on Body Weight of SCWL Cockerels

Age (weeks)	Vitamin A added I.U./kg feed	Mean body weight (g) Lighting regimes (Light:Dark, hr) 10:14		14:10		24:00	
8	6600	(8)[1]	811[a2]	(8)	780[a]	(8)	770[a]
	660	(8)	697[a]	(7)	720[a]	(6)	683[a]
	Ave.		754[a]		752[ab]		732[b]
10	6600	(8)	1077[a]	(8)	1052[a]	(8)	1066[a]
	660	(8)	980[a]	(7)	883[b]	(8)	914[a]
	Ave.		1028[a]		973[ab]		960[b]
12	6600	(8)	1258[a]	(8)	1282[a]	(8)	1201[a]
	660	(8)	1233[a]	(8)	1182[a]	(8)	1158[a]
	Ave.		1245[a]		1232[ab]		1179[b]

[1]Number of observations shown in brackets.
[2]Means within a column or row followed by different small superscripts differ significantly ($P<.05$).

Mean comb weights were expressed as percentages of body weights and are presented in Table 3. As the chicks aged, percent comb weights (PCW) of the A-deficient birds became increasingly greater than those of corresponding controls in all lighting regimes. These differences proved significant ($P<.05$) at 12 weeks for the 14:10 hr groups, and at 10 and 12 weeks for the 24:00 hr groups.

Photoperiod alone (averaged over diet) also influenced PCW (Table 3). Chicks given 14 and 24 hr of light had larger ($P<.05$) percent comb weights than those in 10 hr of light at 10 weeks of age, while only the 14:10 hr group proved significantly greater ($P<.05$) at 12 weeks.

Mean testes weights, expressed as percentages of body weights, are presented in Table 4. Over the time span examined, percent testes weights (PTW) of the mildly A-deficient cockerels became increasingly larger than those of A-adequate birds within respective lighting regimes. These differences became statistically significant ($P<.05$) at 10 and 12 weeks for chicks given 24 and 14 hr of light and at 12 weeks for birds with 10 hr.

TABLE 3

Affects of Dietary Vitamin A, Lighting Regime and Age on Comb Weight as a Percentage of Body Weight of SCWL Cockerels.

Age	Vitamin A	Mean Percent Comb Weight Lighting regimes (Light:Dark, hr)		
(weeks)	IU/kg feed	10:14	14:10	24:00
8	6600	(8)[1] .91[a2]	(8) 1.13[a]	(8) 1.28[a]
	660	(8) .96[a]	(7) 1.75[a]	(6) 1.58[a]
	Ave.	.93[a]	1.43[a]	1.43[a]
10	6600	(8) 1.40[a]	(8) 2.24[a]	(8) 1.68[a]
	660	(8) 1.65[a]	(7) 2.57[a]	(8) 2.80[b]
	Ave.	1.53[a]	2.40[b]	2.24[b]
12	6600	(8) 1.61[a]	(8) 2.63[a]	(8) 1.93[a]
	660	(8) 2.45[a]	(8) 3.53[b]	(8) 2.84[b]
	Ave.	2.03[a]	3.08[b]	2.38[ab]

[1]Number of observations shown in brackets.

[2]Means within a column or row followed by different small superscripts differ significantly (P<.05).

An effect of photoperiod (averaged over diet) was noted in percent testes weight (Table 4). At 10 and 12 weeks of age, cockerels in the 24 and 14 hr treatments exhibited greater (P<.05) PTW values than those given 10 hr of daily light.

Increases in comb and testes weights (alone or as percentages of body weights) in response to a mild A-deficiency have been noted previously (8-11). In addition, the stimulatory effect of day lengths of greater than 12 hours on gonadal growth has also been reported (32, 34, 35). The trends within experiment one in both PCW and PTW indicate that a marginal vitamin A deficiency produces a progonadal effect despite the presence of non-stimulatory (10:14 hr) lighting. Within the 10:14 hr treatments, the marginally deficient chicks exhibited an increasing indifference to the short day length. Ingkasuwan and Ogasawara (17), using Leghorn-type cockerels, found an age-dependent response of the gonads to photoperiods. Chicks subjected to short days (8 hr) began rapid testes development several weeks later than those given long days (14 hr) and attained normal size and function at a later date. The data in the present experiment imply that mildly A-deficient cockerels may reach this photorefractory stage sooner than controls. If this experiment had been continued, this trend may have become more evident.

TABLE 4

Affects of Dietary Vitamin A, Lighting Regime and Age on Testes Weight as a percentage of body weight of SCWL cockerels

Age (weeks)	Vitamin A added I.U./kg feed	Mean percent testes weight Lighting regimes (Light:Dark, hr) 10:14	14:10	24:00
8	6600	(8)[1] .037[a2]	(8) .053[a]	(8) .069[a]
	660	(8) .043[a]	(7) .121[a]	(6) .114[a]
	Ave.	.040[a]	.087[a]	.091[a]
10	6600	(8) .068[a]	(8) .254[a]	(8) .209[a]
	660	(8) .121[a]	(7) .499[b]	(8) .478[b]
	Ave.	.094[a]	.376[b]	.343[b]
12	6600	(8) .057[a]	(8) .604[a]	(8) .437[a]
	660	(8) .510[b]	(8) .794[b]	(8) .824[b]
	Ave.	.283[a]	.699[b]	.630[b]

[1]Number of observations shown in brackets.

[2]Means within a column or row followed by different small superscripts differ significantly (P<.05).

In experiment two the blinded A deficient cockerels had reduced (P<.05) body weights relative to controls (Table 5). Body weight was not affected by light intensity (Table 5). Other researchers (36, 37) have noted that low light intensities (unmeasureable and less than .09 lux, respectively) produced greater body weights than higher levels (64.59 and .18, .48 and .93 lux, respectively) in sighted Leghorn cockerels and broilers, respectively.

Differences in percent comb weights between control and A-deficient chicks were not statistically significant in any of the light intensities (Table 5). However within the A-deficient treatments, cockerels given an intensity of 6.00 lux had greater (P<.05) PCW than those in 37.53 lux.

The effect of vitamin A upon PTW was influenced by the different light intensities, (Table 5). At the two intermediate light intensities (6.00 and 12.08 lux), mildly A-deficient chicks had greater (P<.05) PTW than corresponding controls, while no differences were noted between the diets in the .24 and 37.53 lux treatments.

In experiment two, the results indicated that a mild hypovitaminosis-A state alters primary and secondary sexual development through extraretinal photoreception. Supportive results were reported in a similar experiment by Bukay and Sammelwitz (35). Blinded Golden Buff cockerels were fed either a control

TABLE 5

Affects of Dietary Vitamin A and Light Intensity Levels on Mean Body Weights, Percent Comb and Testes of Blinded SCWL Cockerels

Light intensity (Lux)	Vitamin A added (IU/kg diet)	No. of Chicks	Body weight (g)	Comb, %, of body weight	Testes,% of body weight
.24	6600	4	1281a[1]	1.66ab	.119a
.24	660	4	941b	1.80ab	.259ab
6.00	6600	5	1210a	1.52ab	.106a
6.00	660	5	1023b	2.45 b	.690c
12.08	6600	4	1300a	1.32ab	.070a
12.08	660	4	1052b	1.89ab	.480bc
37.53	6600	4	1256a	1.20ab	.118a
37.53	660	5	966b	0.90a	.075a

[1]Means within a column followed by different small letters differ significantly ($P<.05$).

(6,600 IU/kg retinyl palmitate) or a marginally A-deficient ration (660 IU/kg). Chicks were subjected to constant lighting of 300 or 600 lux intensity. Increasing extraretinal light by plucking head feathers, increased testes weights within the deficient treatments, while no influence was noted in controls.

At all light intensities, chicks fed an A-adequate diet possessed higher ($P<.05$) liver vitamin A concentrations than moderately deficient birds (Table 6). Within the control treatments, 12.08 lux birds had a mean liver vitamin A level which was markedly lower ($P<.05$) than the other groups. The quantity of retinyl palmitate added to the A-deficient ration resulted in near exhaustion of liver stores by 15 weeks of age which were unaffected by light intensity. Decreased plasma vitamin A values substantiate this claim (Table 6). Concentrations of vitamin A in the plasma of control chicks were greater ($P<.05$) than corresponding marginally deficient cockerels.

Liver concentrations of vitamin A are a more reliable indicator of its status than plasma levels. Liver stores are drawn upon when the vitamin is deficient in the diet so that plasma levels remain fairly stable due to rigid regulation by retinol binding protein. When the liver is depleted, plasma levels quickly decline. Stress, increased cellular need or renal loss can also lower vitamin A concentrations in the plasma (38).

TABLE 6

Affects of Dietary Vitamin A and Light Intensity Levels on Liver and Serum Vitamin A Concentrations of Blinded SCWL Cockerels

Light intensity (Lux)	Vitamin A added (IU/kg diet)	No. of Chicks	Mean Vitamin A liver µg/g wet basis	Mean Vitamin A plasma µg/ml
.24	6600	4	71.19a[1]	.520a
.24	660	4	1.52c	.210b
6.00	6600	5	71.45a	.528a
6.00	660	5	1.48c	.164b
12.08	6600	4	44.85b	.476a
12.08	660	4	1.50c	.233b
37.53	6600	4	77.74a	.486a
37.53	660	5	1.41c	.203b

[1]Means within a column followed by different small letters differ significantly ($P<.05$).

Locomotor activity recordings failed to be sufficiently uniform within each treatment to demonstrate any differences. Individual variation in locomotor activity is not uncommon (39). Some of the blinded cockerels did appear to be entrained to the prevailing photoperiod. These recordings resembled those presented by Menaker and Zimmerman (40) for blinded sparrows.

In summation, a moderate vitamin A deficiency was produced in both intact and blinded Single Comb White Leghorn cockerels. This was evidenced by reduced body weights (experiments one and two) and vitamin A concentrations in livers and plasma of the A-deficient chicks (experiment two). However, these birds continued to grow at a rate that paralleled controls (experiment one).

Comb and testes weights, expressed as percentages of body weights, were increased by the vitamin deficiency in the first experiment, while only the latter increased in experiment two. In sighted birds, differences in percent comb and testes weights between control and A-deficient diets became progressively larger as the chicks aged. These trends occurred within all lighting regimes, though in the non-stimulatory (10:14 hr) treatment it appeared somewhat retarded. Therefore, it would seem that the progonadal influence of a moderate vitamin A deficiency was not significantly affected by photoperiod. Blinded cockerels demonstrated an interaction between dietary vitamin A level and light intensity. The two intermediate intensities (6.00 and 12.08 lux) produced the expected differences in percent testes weights between the diets, while extremes in intensity (.24 and 37.53 lux) did not. This implies that vitamin A plays a role in photogonadal response through extraretinal photoreception. A recent study by Deguchi (41) reports that the pineal gland of the chicken, which some propose contains extraocular photoreceptors (26), possesses a rhodopsin-like substance within its cell membranes. This information may partially explain the results of the present experiments, though more research in this area is necessary.

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SOME BEHAVIOURAL EFFECTS OF PRENATAL MARGINAL ZINC DEFICIENCY IN YOUNG RATS

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ABSTRACT

During pregnancy rats were fed either a marginally zinc deficient (18.33 ppm zinc) or a control diet (about 100 ppm). At birth control offspring were fostered to other control dams or cross-fostered to zinc deficient dams. All deficient pups were cross-fostered to control dams. Neonatal mortality was higher in zinc deficient pups. At 14-15 days of age, deficient pups opened their eyes earlier and control pups cross-fostered to deficient dams were heavier and emitted more ultrasonic distress calls than pups from the other groups. They also spontaneously alternated the most at 28-31 days and, along with deficient rats ambulated more than control rats fostered to control dams. At 56-64 days rats in this latter group were lighter than all other rats. For males only, 56-64 day old deficient rats achieved the highest ambulation counts. Control females of this age fostered to control dams showed the lowest female preference for novelty. The results were discussed in the light of complex relationships between prenatal marginal zinc deficiency, postnatal maternal influences, sex of the rats and possible zinc toxicity in control animals.

KEY WORDS : Zinc deficiency - behavior - growth - maternal influences.

INTRODUCTION

Pregnant rats fed severely zinc deficient diets (< 1 ppm) produce offspring showing gross malformations of the central nervous system, skeleton soft tissues, heart, lungs and urinogenital system (1). The incidence of malformations ranges from almost 100% with diets containing 1 ppm zinc or less, to 21% with 6.5 ppm (2). No obvious morphological malformations occur with higher zinc levels although fewer offspring survive to 6 weeks of age with levels as high as 19 ppm (1). If the effects of severe prenatal zinc deficiency on abnormal development of the nervous system are due to depressed nucleic acid synthesis (1), it is conceivable that less severe deficiencies may impair the development of normal brain functioning in the absence of gross morphological malformations. As any impairment might be reflected in behavioral changes, the present study was designed to primarily assess the

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effects of prenatal marginal zinc deficiency on the behavioral development of young rats. A range of tests of unconditioned behavior were applied which involved locomotor activity, timidity and exploration to varying degrees.

MATERIALS AND METHODS

1. Subjects: The dams were 24 random-derived Wistar albino rats aged between 134 and 158 days. They were individually house in 46x29x16.5 cm (LxWxH) cages. To avoid contamination with zinc each cage was constructed from opaque acrylonitrile butadiene styrene plastic with a stainless steel lid. A zinc free steel mesh platform situated 2.5 cm above the floor allowed fecal boli to fall on to underlying sheets of blotting paper thereby reducing the incidence of coprophagy. Distilled water was available from a glass tube projecting from a 500 ml polypropylene bottle which lay in a recess in the lid. The diet was provided as wet mash in a glass container wired to the steel platform.

2. Diets: The marginally zinc deficient diet was based on that described by Hurley and Shrader (3) and comprised 30.0% soya flour (heat-treated for 30 minutes at 106°C to destroy the trypsin inhibiting factor), 55.8% sucrose, 9.5% corn oil, 4.0% salt mix and 0.7% DL-methionine. (The salt mix consisted of 28.40% $CaCO_3$, 10.41% $H_2PO_4)_2.2H_2O$, 30.77% K_2HPO_4, 15.90% NaCl, 11.83% $MgSO_4.7H_2O$, 2.37% $FeSO_4.7H_2O$, 0.22% $MnSO_4.H2O$, 0.08% KI and 0.03% $CuSO_4.5H_2O$). Analyses by atomic absorption spectrophotometry showed the zinc level to be 18.33 ppm. The control diet was produced by adding 0.398 g/100g of $ZnCO_3$ to the salt mix thereby raising the zinc level of the diet to approximately 100 ppm. Every third day both diets were supplemented with a vitain mix designed to ensure that each dam received the following intake (µg) : calcium pantothenate 500; para-aminobenzoic acid 100, riboflavin 100; thiamine HCl 300; pyridoxine 300; nicotinic acid 300; menadione 250; folic acid 6; biotin 2.5; vitamin B_{12} 0.3; choline chloride 10,000; inositol 5,000; alpha-tocopherol 1,000; ascorbic acid 1,000; vitamin A 60; vitamin D 30.

3. Treatment of dams and litters: All dams were fed on the control diet for 20 days. They were then randomly assigned to a control (n = 16) and a zinc deficient group (n = 8). Whereas control dams continued to receive the control diet, dams in the zinc deficient group were fed on the deficient diet. At the same time a male rat was introduced for 3 days into each cage for the purposes of mating. The weight of food consumed and body weight were recorded daily throughout gestation. On the day of parturition, each dam was returned to a normal laboratory diet in dry pellet form. Within 24 hours her litter was counted, weighed and replaced by one from another rat. Thirteen dams in the control group produced litters. Of these 4 were cross fostered to mothers in the zinc deficient group (control ⟶ deficient, C ⟶ D) and 8 were fostered to other mothers in the control group (control ⟶ control, C ⟶ C). For simplicity of design the thirteenth litter was discarded. Only 4 dams in the zinc-deficient group produced litters. These were all cross-fostered to mothers in the control group (deficient ⟶ control, D ⟶ C). All pups were weaned at 25 days of age and caged in groups of 5 or 6 of the same sex.

4. Postnatal behavioral and biometric measures: Measures were recorded at 14-15, 28-31, 56-64 days of age.

a. 14-15 days. When 14 days old, 25 pups were randomly selected from each group, individually weighed and then left for 5 minutes in a 30x30x30 cm clear Perspex container. During this time, numbers of ultra-sonic distress calls (4) were recorded by means of a CFP activity monitor

(model 418/8181) receiver transducer operating at a nominal frequency of 40 KHz connected to a Lafayette counter (model 54417). The following day numbers of these rats with both eyes open were counted. Each animal was then placed into the centre of a 30 cm high 360 cm square Perspex open field with clear walls and a black floor divided into 64 squares by means of white painted lines. A circular 22 watt fluorescent tube was situated 40 cm above the floor of the apparatus. For exactly 3 minutes the number of lines crossed by the hind legs (ambulation) was recorded. Frequencies of ambulation are believed to be negatively correlated with fear or emotionality (5).

b. 28-31 days. When 28 days old 25 rats (12 or 13 of each sex) from each group were weighed and then tested for spontaneous alternation tendencies in a wooden T maze. The apparatus comprised walls 15 cm high and a startbox 15 cm long. The stem was 30 cm long and the two arms were each 40 cm long. Both arms and the startbox could be blocked off from the stem by means of guillotine doors. Each rat was placed in the startbox and allowed access to the rest of the apparatus. It was noted which arm (L or R) the animal entered sufficiently to enable the appropriate door to be lowered behind it. This procedure was repeated a further six times. The number of times the rat alternated its choices of arms (with respect to each immediately preceding trial) out of a maximum of 6 was determined. Alternation is generally accepted as an exploratory response to the more novel arm, namely that least recently visited (6). At 30 days of age open-field ambulation during a 3-minute period was recorded for each subject. The procedure and apparatus was the same as at 15 days except that the floor of the field was divided into 16 squares. On the following day fear or timidity was determined as speed of emergence from a darkened chamber into an illuminated arena (5). The apparatus comprised a 30.5 x 20.5 x 19. 5 cm (LxWxH) Lafayette modular testing unit (model A550) connected to an irregular pentagonal unit of the same height and with a floor area of 900 sq cm (model A563). The units consisted of stainless steel walls with floors made up of 0.5 cm diameter steel rods spaced 1.5 cm apart. One wall in each unit contained a one-way window. Illumination in the pentagonal unit was provided by two 20 watt bulbs in the ceiling. Access from the smaller darkened to the larger illuminated unit was via an electrically operated guillotine door. Each rat was placed in the darkened unit and, 15 seconds later, the door was opened. The time it took the rat to fully enter the illuminated arena was recorded by stopwatch.

c. 56-64 days. Twenty 56 day old animals from each group were weighed and then tested for spontaneous alternation tendencies using the same T maze and procedure as used at 28 days. Open-field ambulation and emergence speed were assessed at 57 and 58 days respectively. The apparatus and procedure were the same as for 30 and 31 days. When the rats reached 60 days of age a programme of testing in one of four 40x40x20 cm (LxWxH) clear Perspex exploration boxes (described in detail elsewhere, 7) was begun for 10 animals in each group. Briefly, each box was divided into four equal cells by partial walls. By means of two opaque Perspex slides inserted into gaps in an opaque Perspex wall it was possible to confine each rat to one half of a box. Each box was situated within an illuminated, sound-attenuated air-conditioned cubicle. The animal was observed by means of a one-way window in the front wall of each cubicle. Each animal was confined to one half of a box for 60 minutes. The slides separating the two halves were then removed and for exactly 10 minutes, it was noted every 5 seconds if the rat was in the previously inaccessible novel half (novelty preference). The total number of cells entered was also recorded. Novelty preference provides an unequivocal index of exploratory behavior relatively uncontaminated by locomotor activity (8).

RESULTS

1. Observations of pregnant dams: The mean daily intake of food during mating and gestation was 9.5g for pregnant dams fed the control diet and 10.3g for those fed the zinc deficient diet. According to a t-test this difference was not significant ($t = 1.74$, $df = 15$). Mean bodyweights one day before parturition were 283.2 and 283.3g for pregnant rats fed control and deficient diets respectively. This difference was not significant ($t < 1$).

2. Observations of newborn litters: The 13 out of 16 dams on the control diet and 4 out 8 on the deficient diet who produced litters were compared by means of a Fisher exact probability test. The proportions were not significantly different ($p = 0.11$). Mean litter size was 8.8 pups for control and 7.0 pups for deficient dams. Mean average weight of pups was 5.1g for control and 4.8 for deficient rats. In neither case was the difference significant ($t < 1$). Nine of the 114 pups (7.9%) born to control dams died within 48 hours of birth compared with 8 of the 32 (25%) born to zinc deficient dams. According to a chi-square test the difference between these proportions was significant ($\chi^2 = 5.54$, $df = 1$, $p < 0.02$).

3. Observations of offspring at varying stages of development: Unless specified otherwise, all data were analysed by one-way analyses of variance at 14-15 days of age and two-way analyses of variance (groups x sex) thereafter. Significant effects were followed by individual t-test comparisons using df for error and MS error as variance estimates. Bodyweight and behavioral results can be seen in Table 1.

a. 14-15 days. Significant group effects occurred with bodyweight and ultrasonic calls. In both cases C⟶D rats were heavier and called more often than rats from the other two groups who did not differ significantly from each other. According to a chi-square test there was an overall between-groups difference in the number of rats observed with both eyes open on day 15 ($\chi^2 = 13.84$, $df = 2$, $p < 0.001$). Further tests showed that although they did not differ from each other, groups C⟶C (14/25) and C⟶D (19/25) both differed significantly from group D⟶C (25/25).

b. 28-31 days. There were significant group effects for alternation and ambulation. Rats from group C⟶D alternated more often and C⟶C rats, crossed fewer lines in the open field than rats in the other two groups who did not differ significantly from each other in either case. Females (mean = 74.5) crossed more lines than males (mean = 64.5, $F [1,69] = 4.60$, $p < 0.05$).

c. 56-64 days. Significant groups effects were evident with bodyweight and rearing in an exploration box. Rats from group C⟶C were significantly lighter than rats from the other two groups. Males (mean = 201.1g) were heavier than females (mean = 152.6g, $F [1,54] = 5.58$, $p < 0.01$). Females emerged faster (mean = 43.8 sec, male mean=69.9 sec, $F [1,54] = 7.20$, $p < 0.01$) and alternated more frequently (mean = 66.0%, male mean = 52.7%, $F [1,52] = 6.66$, $p < 0.01$) than males. There was also a significant sex difference for ambulation which is more appropriately considered in the light of a significant groups x sex interaction ($F [2,54] = 4.67$, $p < 0.05$). Individual t-tests revealed no significant between-groups differences for females (means, C⟶C = 74.2, C⟶D = 77.4, D⟶C = 66.4). However, D⟶C males (mean = 75.5) crossed more lines than males from either the C⟶D (mean = 64.5) or C⟶C (mean = 50.0) groups. There was also a near-significant groups x sex interaction for novelty preference ($F [2,24] = 3.30$, $p < 0.1 > 0.05$) which arose from significantly lower scores for

TABLE 1

Bodyweight and Behavioral Data (means ± standard deviations) for Control Rats Fostered to Control Dams (C→C) Control Rats Cross-fostered to Zinc Deficient Dams (C→D) and Deficient Rats Cross-fostered to Control Dams (D→C), and F Ratios

MEASURE	GROUP			F (df)
	C→C	C→D	D→C	
a. 14-15 days				
Bodyweight (grams)	24.4(± 3.2)	27.3(± 1.6)	25.6(± 3.9)	5.64 (2,73)**
Ultrasonic calls (calls/minute)	41.8(±27.4)	59.2(±30.0)	40.3(±26.6)	3.16 (2,73)*
Ambulation	24.8(±17.3)	21.5(±14.7)	16.9(±13.0)	1.67 (2,73)
b. 28-31 days				
Bodyweight (grams)	66.6(± 8.9)	69.6(± 5.3)	66.6(±10.7)	0.79 (2,69)
Alternation (per cent)	44.0(±18.2)	59.3(±24.09)	48.0(±21.8)	3.15 (2,69)*
Ambulation (lines cross)[a]	58.3(±18.3)	70.7(±15.3)	79.8(±22.4)	7.78 (2,69)**
Emergence speed (seconds)	109.7(±58.7)	74.2(±29.6)	88.7(±64.1)	2.19 (2,56)
c. 56-64 days				
Bodyweight (grams)[a]	166.6(±23.3)	184.6(±31.0)	189.4(±34.9)	5.58 (2,54)**
Alternation (per cent)[a]	52.5(±24.3)	63.0(±19.6)	61.7(±14.0)	1.53 (2,52)
Ambulation (lines cross)[a,b]	60.9(±22.3)	71.0(±17.1)	71.9(±14.8)	1.69 (2,54)
Emergence speed (seconds)[a]	57.3(±26.2)	61.3(±55.1)	55.9(±26.6)	0.22 (2,54)
Novelty preference (5-second observations)[b]	75.4(± 7.2)	83.3(±23.9)	83.2(± 8.4)	2.04 (2,24)
Cells entered	42.8(±17.3)	36.7(± 8.9)	46.1(±15.7)	0.95 (2,24)

* $p < 0.05$ ** $p < 0.01$

a sex difference significant (see text)
b sex x group interaction significant (see text)

C→C females (mean = 72.2) than for those in the other two groups (means, C→D = 90.8, D→C = 87.2), and a lack of any groups effect for males (means, C→C = 78.6, C→D = 75.8, D→C = 79.2).

DISCUSSION

Dams fed on the marginally zinc deficient diet did not differ from those fed on the control diet in terms of daily food intake, weight-gain, fertility, litter-size or average weight of pups. However, neonatal mortality was higher in pups of zinc deficient mothers. This finding is in accord with the observation that a prenatal diet containing 18 ppm zinc decreased survival to 6 weeks of age (1). Although systematic observations were not carried out at the time of birth it was nevertheless apparent that, as noted earlier (9) zinc deficient dams experienced considerable difficulty with delivery.

From 14 days of age onwards it is clear that the effects of prenatal zinc deficiency were more complicated than anticipated. At 14 days control pups cross-fostered to zinc deficient dams were heavier and, as shown by their ultrasonic calls, appeared more distressed when removed from the nest than either control or deficient rats reared by control dams. Because of the survival value of ultrasonic calls, control pups cross-fostered to deficient dams may have been exhibiting more adaptive behavior than pups in the other group. This suggests that the postnatal influence of dams fed the zinc deficient diet during pregnancy was to somehow facilitate growth but at the same time increase susceptibility to the stress of being removed from the nest. Whether this was due to zinc deficiency-induced modification of maternal behavior (9) or to postnatal zinc deficiency in the colustrum and milk remains to be determined. The latter possibility seems unlikely in view of the fact that, at this stage of development, prenatal zinc deficiency seemed behaviorally ineffective. However, such deficiency markedly accelerated eye-opening (at least as observed on day 15 after birth).

During days 28-31, the earlier significant bodyweight difference was no longer apparent. Both prenatal zinc deficiency and possible postnatal zinc deficient maternal effects produced higher levels of ambulatory activity but only the latter influence increased spontaneous alternation. One might be tempted to suggest that both manipulations decreased emotional reactivity if it were not for the lack of a parallel effect on emergence speed. Instead it is more likely that separate mechanisms (other than emotionality) underlying ambulation and alternation were affected.

From day 56 onwards effects of marginal prenatal zinc deficiency became more apparent. Such effects were similar to those resulting from the earlier cross-fostering of control pups to formerly zinc deficient dams. It appeared that the behavioral outcomes of the experimental treatments were partially dependent on the sex of the animals. Both prenatally zinc deficient rats and normal animals cross-fostered to zinc deficient dams were heavier and reared less in an exploration box than controls. These experimental treatments also increased open-field ambulation for males and novelty preferences in an exploration box for females.

The inclusion of fostering procedures in the present study revealed the profound influence that dams fed the zinc deficient diet during pregnancy can have on the development of normal pups. However, rather than this influence being detrimental, it could be seen as "beneficial" i.e., heavier weight and more ultrasonic signalling at 14 days; more locomotor activity and exploration of novelty (alternation, 6) at 28-31 days; heavier weight, more locomotor activity (for males only) and higher preferences for novelty (for females only) at 56-64 days. Similarly, prenatal zinc deficiency effects appeared developmentally "beneficial" i.e., earlier opening of eyes; more locomotor activity at 30 days; heavier weight, more locomotor activity (for males only) and higher preferences for novelty (for females only) at

56-64 days. It can not yet be determined why these two general influences should appear to benefit development. The apparent advantages of prenatal zinc deficiency may in part have arisen from mild toxicity of the approximately 100 ppm zinc in the control diet (I.E. Dreosti, personal communication, September, 1980) even though this level is usually regarded as within the normal range (3,10). This possibility is the subject of current research in this laboratory. In view of their higher neonatal mortality, perhaps only excessively robust, adaptable offspring of dams fed the zinc deficient diet survived the prenatal manipulation. Why this influence should take longer to become evident than a postnatal maternal influence remains to be explained. Zinc deprived dams display inadequate maternal attentiveness (9) therefore, control pups in the present study cross-fostered to zinc deficient dams may have been denied sufficient maternal care. If this lack of attention were stressful the rats may have been "immunized" against later stressors in the manner proposed by Levine (11). Reduced stressfulness of the behavioral tests applied could then have resulted in superior performance. Some limited support for this idea is found in the observation, that before weaning (14-15 days), these rats appeared more stressed than other rats when removed from the nest as indicated by their higher frequency of ultrasonic calls. However, their heavier bodyweights at this age are inconsistent with a disrupted maternal behavior hypothesis. Further research is obviously needed.

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NONIMPROVEMENT OF HEALING OF JEJUNAL ANASTOMOSES BY EXOGENOUS AMINO ACIDS IN PROTEIN DEPLETED RATS

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ABSTRACT

The healing rate, as measured by bursting strength in 5 days of jejunal anastomoses in protein depleted rats is not improved by a) feeding stock diet rather than protein free diet following surgery or b) infusing a total parenteral nutrition (TPN) solution known to improve the healing rate of jejunal anastomoses in normal rats rather than either a standard TPN solution or 5% glucose.

Key Words: Healing • protein depleted • amino acids

INTRODUCTION

We have previously shown that the healing rate, as measured by bursting strength in five days, of jejunal anastomoses in normal rats, is significantly increased when glutamine, additional alanine and choline are added to a standard total parenteral nutrition (TPN) solution compared to the standard TPN solution itself (1). The value of nutritional support in the long-term management of intestinal fistulas, inflammatory intestinal disease and following massive resection of the small intestine has been established. However, the value of short periods of hyperalimentation in the prophylaxis of complications in malnourished patients undergoing extensive surgical procedures is more controversial (2). Steiger et al. (3) found that infusing protein depleted adult female rats with 30% dextrose and 5% amino acids increased the healing rate of colonic anastomoses 34% when compared to similar rats infused with 5% dextrose. On the other hand, Irvin and Hunt (4) found the bursting pressure and bursting tension of a colonic anastomosis to be the same following surgery regardless of whether adult male rats were fed a protein free diet for three to five weeks before and one week following surgery or whether they were weight restricted on stock diet for the same time. Irvin (2) found no difference in the breaking strength of colonic anastomoses in male rats fed a protein free diet 8 weeks before and one week following surgery than in the bursting strength of colonic anastomoses from rats fed the same protein free diet supplemented with 3% amino acids in drinking water one week before and one week after surgery. Sandberg (5) found that the tensile strength of skin wounds in adult male rats fed a restricted amount of stock diet such that they lost 17% of their body weight in the seven days following surgery was the same as that of ad libitum fed rats whose weight varied only slightly.

We have examined the ability of both stock diet and a TPN formula, which improves the healing rate of jejunal anastomoses in normal rats (1), to improve the healing rate of jejunal anastomoses in malnourished rats and report the results in this paper.

TABLE 1
Composition of Total Parenteral Nutrition Solutions

Component	Standard TPN (ml)	CA (ml)
Travasol, 5.5%*	500	400
Electrolytes	**	**
Glucose, 50%	500	400
Alanine, 17.5%		100
Glutamine, 3.5%		100
Calcium Gluconate, 10%	5	5
Choline Chloride, 22.4%		3
MVI Vitamin Concentrate†	1	1
Mg N/day	184	283
Cal/day‡	41.0	36.1

*Travasol, 5.5%: Each 100 ml contains L-leucine, 340 mg; L-phenylalanine, 340 mg; L-methionine, 318 mg; L-lysine, 318 mg; L-isoleucine, 263 mg; L-valine, 252 mg; L-histidine, 241 mg; L-threonine, 230 mg; L-tryptophan, 99 mg; L-alanine, 1.14 g; glycine, 1.14 g; L-arginine, 570 mg; L-proline, 230 mg; L-tyrosine, 22 mg; acetate, 3.5 mEq; chloride, 2.2 mEq; sodium 0.3 mEq.

**Electrolytes: 1.36 ml of 80% $MgCl_2 \cdot 6H_2O$, 5.60 ml of 20% NaCl, 6.52 ml of 40% K_2HPO_4 and 6.17 ml of 35% sodium acetate $3H_2O$ added to each formula; all solutions w/v.

†MVI vitamin concentrate: Each 1 ml contains: 100 mg ascorbic acid, 2000 IU vitamin A; 200 IU vitamin D; 10 mg thiamine·HCl; 2 mg riboflavin-5-phosphate; 3 mg pyridoxine·HCl; 20 mg niacinamide; 5 mg dexpanthenol; 1 IU vitamin E.

‡Non-protein calories.

METHODS

Male Sprague-Dawley derived rats[1], weighing 300-400 g, were placed on a protein depletion diet (protein-free, (USP XV)[2] for three weeks during which they lost an average of 83 g body weight. The rats were fasted overnight, anesthetized with pentobarbital and a jejunal anastomosis performed according to the procedure of Lambert (6). The jugular vein of rats to be infused was catheterized at the same time. The rats were infused continuously with nutrient solutions (7), Table 1, or 5% glucose at a rate of 1.7 ml/h (3). The rats were able to move about the cage during infusion with the use of the "curtain-wire" technique of Dalton et al. (8). Five days following surgery the rats were sacrificed by decapitation and a section of jejunum removed which included the anastomosis. One end of the jejunum was fastened to a manometer approximately 1 cm from the anastomosis. The other end was tied off 1 cm from the anastomosis. The jejunum was placed in a beaker of saline and the pressure increased approximately 100 mm Hg per minute until the intestine burst. The pressure at which bursting of the intestine occurred was recorded as the bursting pressure.

RESULTS

Five days were chosen as the test time point from a curve of bursting strength versus time. In jejunal anastomosis in normal rats on stock diet, the bursting strength falls from the first day to the second and third day. It begins to rise on the fourth day and by the fifth day is just short of normal strength. On the sixth day the bursting strength of the anastomosis exceeds the bursting strength of a normal jejunum. Similar time curves have been observed in the bursting strengths of colonic anastomoses (9).

The bursting strengths of jejunal anastomoses of the five groups of rats five days following surgery are shown in Table 2. The bursting strength of a jejunal anastomosis is a quantitative measure of healing (9) and serves to evaluate the effect of various nutrient regimens on the healing process.

Jejunal anastomoses in non-restrained protein depleted rats healed at the same rate regardless whether they received stock diet or protein depletion diet following surgery. Jejunal anastomoses in infused rats healed at the same rate regardless whether they were infused with a standard TPN solution, a TPN solution which increases the healing rate in normal rats (1), solution CA, or 5% glucose.

[1]Biolab, White Bear Lake, Minnesota.
[2]ICN Nutritional Biochemicals, Cleveland, Ohio.

TABLE 2
Bursting Strengths of Jejunal Anastomoses, Calorie and Water Consumption of Protein-Depleted Rats Five Days Following Surgery

	Bursting strength (mm Hg)*	N/day (mg)	Total** cal/day (cal)	H_2O/ day (ml)
Non-restrained				
Normal rat stock diet and water ad lib.	255.3±7.6 (14)a	698	65.3	25.4
Stock diet and water ad lib.	221.6±11.1(15)b	669	62.6	27.0
PD diet and water ad lib.	218.1±6.6 (15)b	0	44.6	14.2
Restrained, intravenous				
Standard TPN	202.2±6.8 (20)a	184	45.6	40.8
CA	215.9±7.3 (15)a	283	43.2	40.8
D_5W	216.0±12.1 (5)a	0	7.4	40.8

*Mean±SEM(N). Means with a letter in common are not significantly different at the 5% level, Student t-test. PD=protein depletion diet.
**Including protein/amino acid calories.

DISCUSSION

The healing of jejunal anastomoses in protein depleted rats was equally supported by a protein free diet and by a stock diet containing 22.5% protein, Table 2. Not only did the rats fed the protein depletion diet following surgery receive no protein, they also consumed fewer total calories and water. Thus, the exogenous protein and additional energy must have been of no use to these particular rats with regard to healing their jejunal anastomoses. The amino acids required to heal the anastomosis must have been scavenged from the surrounding tissues and the lower calorie and water intake must have been sufficient to supply energy for healing the anastomoses and maintain fluid balance.

The harness fitted around the chest of rats infused by the "curtain-wire" technique used in these experiments induces restraint stress in normal rats which reduces the healing rate (10). Thus, the healing rate of non-restrained rats cannot be compared with the healing rate of restrained rats. Infusion of solution CA, a modification of the standard TPN solution in which glutamine, additional alanine and choline are added to improve the healing rate of jejunal anastomoses in normal rats by 18% (1), improved the healing rate of protein depleted rats by only 7%, Table 2, a non-significant increase. The healing rate supported by solution CA was no better than that supported by 5% glucose. Thus, the infusion of exogenous amino acids and energy greater than that supplied by 5% glucose were of no value with regard to increasing the healing rate of jejunal anastomoses.

An explanation of the lack of response of the healing of jejunal anastomoses in protein depleted rats to enteral protein or parenteral amino acids may be found in the inability of the enzymes present to utilize the additional amino acids. A negative nitrogen balance does not necessarily interfere with wound healing. The concept of primacy of the wound is real and is demonstrated by the ability of even cachectic patients to attain wound healing, but at the expense of other tissues (11).

The data collected in this experiment indicates that the amino acid pool utilized for healing the jejunum is of sufficient size, even after three weeks of protein free diet, that supplementation by enteral protein or parenteral amino acids is of no assistance to the healing process. Thus, the available amino acids must saturate the repair system after three weeks of protein free diet.

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COMMENTS ON BREAST FEEDING AND GROWTH IN AUSTRALIAN INFANTS

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The article by Nancy E. Hitchcock and coworkers ("Dietary energy and nutrient intakes and growth of healthy Australian infants in the first year of life," Nutrition Research, Vol. 2, pp. 13-19, 1982) gives the mistaken impression that breast feeding is associated with marked growth retardation. The authors state in the abstract that "Body weight increases in this group [their study sample of partially breast-fed infants] were considerably below [my emphasis] those reported from infants in Australia when artificial feeding prevailed" (1). Also, the authors write that growth data over the 12 months resemble those reported in Australia almost 50 years ago when breast feeding prevailed" (1). The purpose of this letter is to point out that the Australian infants studied by Hitchcock show normal growth patterns and that in their sample, breast feeding cannot be said to retard physical growth.

The mean lengths of the Australian infants are compared in Table 1 to the NCHS growth norms (which the World Health Organization and others have been promoting as the preferred international standards). The mean lengths are very similar to the NCHS 50th percentile values and, given the 95% confidence interval of the sample means, the Australian and NCHS populations cannot be said to be different in regards to mean lengths. Mean lengths are equivalent to percentile values slightly below the 50th percentile in the case of boys and slightly above the 50th in the case of girls.

A similar analysis is shown in Table 2 for weight. In addition, weights from a 1933 and a 1964 study are included. These two samples were used by Hitchcock et al. (1) as examples respectively of the time when breast feeding was prevalent (1933) and when bottle feeding (1964) prevailed. The mean weights of the 1980 Australian sample are nearly identical to the NCHS 50th percentile values. The mean weights of the 1964 sample, on the other hand, are clearly greater than the NCHS, 1980, and 1933 values. Also, the mean weights of the 1933 and 1980 samples are similar, particularly in the case of girls.

In North America, the NCHS norms are replacing the Iowa-Harvard norms which are based on growth data from the '30s and '40s. Interestingly, the new and the old norms are not that different at the 50th percentile values. The Australian 1933 sample also shows modern patterns of growth but this might be the result of selection bias. The 1933 sample was drawn from amongst the regular attenders to several "baby health centres." Irregular attenders, children who had been ill at any time during infancy, twins and prematures[a] were excluded. More importantly, the sample selected was "only. . .those who the sisters were convinced had run a natural, normal course, whether they were slightly above or below the average weight." Thus, one wonders about the extent to which the sample selected was representative of the populations served by the baby health centers. Finally, Clements (4) did not comment on

[a] Hitchcock et al. (1) excluded infants with birth weights less than 2500 g. This may partly explain the relatively high birth weights reported.

TABLE 1

Length of Australian Infants

Age (months)	NCHS 50th percentile[a]	Australian mean[b]	95% confidence interval of mean[c]	Australian percentile score[d]
		Boys		
Birth	50.5	51.6	± 0.4	71
3	61.1	60.7	± 0.4	41
6	67.8	67.5	± 0.6	46
9	72.3	72.1	± 0.6	47
12	76.1	75.9	± 0.6	47
		Girls		
Birth	49.9	50.7	± 0.4	68
3	59.5	59.3	± 0.4	47
6	65.9	65.9	± 0.4	50
9	70.4	70.6	± 0.4	53
12	74.3	74.9	± 0.4	58

[a] From Hamill et al. (2).

[b] From Hitchcock et al. (1).

[c] From Hitchcock et al. (1). The 95% confidence interval is 1.96 x S.E.M.

[d] Obtained by interpolation of the percentile tables given by Hamill et al. (2).

infant feeding patterns in the 1933 sample.

The 1964 sample is the heaviest. Relative to the NCHS data, mean weights are equivalent to the 82 and 81 percentile values for boys and girls respectively at 12 months of age. Very likely, weight for lengths are excessive as well. The 1980 sample on the other hand, has weight for length values which are near the 50th percentile values.

In summary, the 1980 sample studied by Hitchcock et al. (1) appears to be growing normally and breast feeding cannot be said to be associated with growth retardation. Children from the 1980s grew like children from 1933 but these may be unrepresentative of breast-fed Australian children of that time. Finally, the 1980 sample is much lighter than the 1964 sample which is clearly overweight.

TALBE 2

Comparison of 1933, 1964, and 1980 Infant Mean Weights

Age (months)	NCHS 50th percentile[a] (kg)	Mean weights (kg)			Mean percentile score[e]		
		1980[b]	1964[c]	1933[d]	1980	1964	1933
			Boys				
Birth	3.27	3.56 ± .08	3.51	3.69	70	66	82
3	5.98	5.92 ± .16	6.73	5.85	48	79	45
6	7.85	7.85 ± .18	8.84	7.53	50	85	38
9	9.18	9.11 ± .20	10.17	8.86	47	82	37
12	10.15	10.01 ± .22	11.20	9.89	45	82	40
			Girls				
Birth	3.23	3.47 ± .08	3.45	3.37	71	69	61
3	5.40	5.61 ± .12	6.22	5.64	61	85	62
6	7.21	7.44 ± .16	8.18	7.37	59	85	56
9	8.56	8.56 ± .18	9.40	8.58	50	79	50
12	9.53	9.50 ± .10	10.48	9.58	49	81	52

[a]From Hamill et al. (2).

[b]1980 mean and 95% confidence interval (i.e., 1.96 x S.E.M.) values from Hitchcock et al. (1).

[c]1964 values as given in Hitchcock et al. (3).

[d]1933 values from Clements (4). Three six, nine, and twelve months were taken to represent 13, 26, 39, and 52 weeks respectively.

[e]Calculated by interpolation from the percentile tables given by Hamill et al. (2).

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REPLY TO DR. MARTORELL

Dr. Martorell is, indeed, "mistaken" if he interprets our findings (1) to suggest that breast-feeding is associated with growth retardation. We must stress that nowhere in our paper did we claim that breast-feeding slows physical growth in infancy. We agree with his assessment that the 1964 (Australian) figures quoted are heaviest and considerably above the median weight for age figures from the NCHS data.

Since reporting our findings to "Nutrition Research" we have completed a prospective study of 686 infants attending metropolitan and rural child health centres in Western Australia (2). These infants had similar growth patterns to subjects in the Perth Growth Study with significantly smaller weight gains in their first year than figures obtained in the early 1970s to compile the Australian National Health and Medical Research Council's "Charts and tables of heights, masses and head circumferences of infants and children" (3). The results from these studies are not significantly different from the NCHS 50th centile values, reinforcing Dr. Martorell's interpretation that Australian infants are now showing normal patterns of growth, almost identical to those seen in the United States. Our findings support recent proposals that the NCHS figures should be used in international comparisons of growth. They also argue strongly against the development of new, national growth standards in Australia based on yet another expensive, large-scale, cross-sectional anthropometric survey (4).

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SUMMARIES OF CONFERENCES

AMERICAN SOCIETY FOR CLINICAL NUTRITION

The Twenty-Second Annual Meeting of the American Society for Clinical Nutrition (ASCN) was held at the Sheraton-Washington Hotel on May 7, 8, and 10. At the business meeting Dr. SAM FOMON turned over the Presidentship of the Society to Dr. HAROLD SANDSTEAD. It was announced that Dr. IRWIN ROSENBERG had been chosen by the membership as the President-elect for 1983.

This year's symposium topic was Assessment of the Evidence Relating Certain Vitamins and Minerals to Health and Disease in the Elderly. It was sponsored, in part, by the National Dairy Council and chaired by Drs. R.S. RIVLIN AND E.A. YOUNG. The keynote speaker was Dr. R.N. BUTLER, Director of the National Institute on Aging, Bethesda. He outlined the various research concerns about the nutritional health of the elderly and how they were to be addressed. The ASCN had received an NIH grant to develop a state-of-the-art report on the issues of mineral and vitamin nutriture in the aged. Panels had been formed, and the spokespersons for each panel constituted the program of the Symposium. Each presentation had a common format in which the best available evidence on customary intakes in the elderly (most taken from the Ten-State and HANES surveys), the current Recommended Dietary Allowance for the nutrient, the special stresses of aging, and the estimated prevalence of nutritional deficiencies was discussed. The micronutrient topics and the discussants were as follows: Calcium (R.P. HEANEY), Iron (S.R. LYNCH), Zinc (H.H. SANDSTEAD), Folate (I.H. ROSENBERG) and Thiamin (F.L. IBER). Among the important insights was the great restriction in sun exposure and intrinsic vitamin D synthesis in older individuals and the optimistic fact that evidence now suggests that increasing calcium intake may indeed prevent, or retard the loss of bone mineral mass. It was reported that there is serious interest in the possible role of thiamin nutriture in Alzheimer's disease. There was no indication that iron or folate deficiency were more prevalent in the elderly than in younger populations in the U.S., and some suggestion that they might be *less* frequent. The picture with zinc nutriture in the elderly is unclear and diagnostic assessment technology needs to be refined.

There were 33 short 10-minute presentations with slides in three scientific sessions. The ASCN Student Award went to R.J. SIRAGUSA of Gainesville who, with his mentor, J.J. CERDA had investigated the extent of methanol production from bacterial degradation of pectin, an obvious concern for those who favor fiber supplementation with citrus fruits. In the study entitled Methanol Production from the Degradation of Pectin by Human Colonic Bacteria, fecal bacteria could produce the alcohol, but in concentrations far below those considered to be toxic.

Five papers on the program addressed issues that generated real novelty and clinical excitement. The first by R.L. FISHER et al at Yale, Peripheral Parenteral Nutrition in Adults: Used or Abused?, demonstrated the common and legion mistakes in the prescription, management, monitoring, and delivering of peripheral parenteral nutrition. Strict criteria appear to be needed, or this will

Zinc has now become an old faithful at these meetings. This year, H. SPENCER from Chicago in Effect of the Calcium Intake on the Inhibitory Action of Zinc on Calcium Absorption showed that pharmacological doses of zinc, 154 mg daily, inhibited calcium absorption by human volunteers when fed a low-calcium (230 mg/day) but not an adequate-calcium diet. In a rat model, M.B. ESSATARA and co-workers answered in the affirmative the question posed in their study Is Zinc Deficiency Anorexia Mediated Through the Endogenous Opiates? The search for the unequivocal laboratory assessment technique for human zinc nutriture has eluded many investigators for decades. A.S. PRASAD and Z.T. COSSACK demonstrated a good correlation between neutrophil zinc levels and other indices of zinc status in patients with sickle cell anemia. Dark adaptation and serum testosterone were among the parameters with which white cell zinc was closely correlated in the paper Neutrophil Zinc: An Indicator of Zinc Status in Man.

Two investigations into infant and child-feeding focused on the role of lactose. In Mineral Absorption from Soy-based and Milk-based Infant Formulas, E.E. ZIEGLER et al from Iowa replaced sucrose with lactose in a soy-based formula diet and found that calcium and phosphorus retention was better than that of milk with an equivalent lactose content. The nature of the protein source did not influence absorption and retention of copper, iron, magnesium and zinc. N.W. SOLOMONS and colleagues in Guatemala concluded that Intact Whole Cow's Milk is Equivalent to Lactose-Hydrolyzed Milk for the Early Recovery from Protein-Energy Malnutrition. Except for more diarrhea in the first week of admission - diarrhea that did not influence nutrient absorption - all of the parameters of growth, clinical recovery, and nutrient retention were identical in children with kwashiorkor fed either form of milk as the base of the recovery diet.

Recent years have witnessed a surge of interest in the quantitative and metabolic aspects of obesity. The ASCN meeting again reflected this trend. In a continuation of the theme presented at these meetings the previous year, DIETZ et al in Boston, using elegant procedures of body-pool marking and turnover assessment involving uniformly labelled ^{13}C-glucose and ^{15}N-lysine, were able to study Interrelationship of Glucose Production and Protein Catabolism during Hypocaloric Dietary Therapy. Two isocaloric weight-reduction diets - one with glucose, the other with fat (ketogenic) - as the exogenous energy sources were fed. Rates of glucose production in neither instance were found to be related to protein catabolism. A multi-national collaborative effort of researchers from Lausanne, Switzerland, Burlington, Vermont, and Phoenix, Arizona focused on an Indian community to address the issue Obesity in the Pima Indians: Is there a Thrifty Gene? By "thrifty gene", R.J. DANIELS inquired as to whether or not an altered metabolic response to a meal with greater energy retention is the cause of the pattern of obesity in this tribal group. Indeed, in obese Indians, the thermic response to a meal was blunted, as was the resting metabolic rates during overfeeding, as compared to lean Caucasian subjects studied elsewhere. Whether this is different from the leaner members of the tribe, and whether this is a cause or effect of the obesity, are questions that demand further address before the hypothesis can be tested in full.

Issues of feeding and obesity concerned the other two papers in this topic area. L.A. LEITER and E.B. MARLISS in Toronto studied the response to different refeeding schedules following a month-long, total therapeutic fast in 15 non-diabetic, obese patients via Stepwise Reintroduction of Carbohydrate during Refeeding after Prolonged Fest. They noted that slow reintroduction minimized fluid retention and provided a moderate clearing of ketosis. R.L. ATKINSON at Virginia, noting previous demonstration that intestinal by-pass and ileal transposition to the jejunum both decrease food intake in rats, asked the *mechanistic* question of whether Nutrients in the Ileum Decrease Food Intake in

just become another way in which physicians will be able to starve their patients (daily caloric intakes were 20 kcal/kg) while believing they are being nourished, at an average cost of $627.27 for an 8-day course. However, the use of energy sources that may improve the potential of peripheral TPN was presented by E.A. YOUNG and colleagues from San Antonio in The Effect of Exercise on the Metabolism of Intravenously Infused Glucose Oligosaccharides in Man; exercise did not affect metabolism, but the important fact was the retention and metabolism of at least half of the infused load of glucose oligosaccharides. A novel and innovative approach to the estimation of lean-body mass was provided from the group at Columbia University. E. PRESTA and co-workers in Measurement of Electrical Conductivity of the Human Body: A New Method for Estimation of Lean Body Mass used the fact that the aqueous, but not anhydrous (fat), tissue conducts electricity and passed their obese subjects on a conveyer through the coils of a massive solenoid. The results correlated well with estimations of total body water and total body potassium, but the test-retest reproducibility was on the order of only 10%. A dismal failure in one form of treating obesity was reported in a critical review by W.W. FALOON et al of Rochester in Gastric Surgery for Obesity: Poor Experience with Conversions from Jejunoileal By-Pass and Reoperation. Despite optimism about gastroplasty as a procedure with less morbidity and greater success rates, the experience at Rochester would indicate that as initial therapy, or especially as a second procedure, gastric surgery for obesity has an unacceptable failure rate. A new, psychopathological adaptation to avoid obesity in adolescence was described by pediatricians and psychiatrists at Cornell in Fear of Obesity - A New Syndrome of Delayed Growth and Sexual Development in Adolescence, presented by M. PUGLIESE et al. The syndrome, unlike anorexia nervosa, had a male predominance of 7 out of 11 patients, and was characterized by growth retardation, underweight and delayed puberty through chronic, voluntary reduction in intake. More is to be expected from active inquiry in all of these areas in the near future.

Several papers dealt with alcohol. In elegant *in vitro* studies done on duodenal tissue taken at routine endoscopy, A. HOYUMPA and colleagues at Vanderbilt in Effect of Ethanol on Glucose Uptake by Human Duodenal Mucosa demonstrated a sodium-dependent, ethanol-induced glucose transport defect; the effect of alcohol seems to be inhibition of Na-coupled glucose movement. J. PINTO et al in Differential Effects of Ethanol upon Bioavailability of Various Forms of Riboflavin found the uptake of vitamin B_2 to be depressed in rats when given with 20% ethanol; the food form (flavin-adenine dinucleotide) was more vulnerable than the vitamin-pill form (riboflavin). When the monkeys who had been on a 4-year study of 50% substitution of ethanol in dietary energy at the Davis California Primate Research Center were sacrificed, C.L. KEEN and co-workers were able to show reduced concentrations of copper, zinc and copper-zinc superoxide dismutase activity, but *increased* manganese-containing enzyme. This corresponded with enlarged mitochondria and elevated hepatic manganese. The authors speculated that this might be in compensation for the reduction in the other species of dismutase. The paper was entitled Effect of Chronic Ethanol Feeding on the Activity of Superoxide Dismutase in Monkeys.

Trace elements, as usual, were represented on the program in the form of several studies in the fields of selenium and zinc. Both studies with selenium by M. SHILS and O.A. LEVANDER dealt with the delivery of this trace mineral in total parenteral nutrition. In Selenium Stability in TPN Solutions they showed that the old formulation of parenteral multivitamin solution (MVI) precipitated selenium out of solution. The factors were abscorbic acid, elemental copper, and other uncharacterized interactions. Newer, MVI-12 does not have this effect. Investigation of patients on home parenteral nutrition in Selenium Levels in Long Term TPN Patients showed circulating levels had fallen below 1 ug/dl in two-thirds of subjects with a greater than 3-year course. Daily supplementation of the solutions with 50 ug of selenium as the selenite, with the MVI-12 vitamin-mineral preparation, lead to steady increases in selenium levels.

Rats. Study was performed by direct injection of solutions into the terminal ileum of the animals. Partially-digested foods, but not pure nutrient (elemental) solutions, were found to effect a reduction in spontaneous food intake. The revelance of this observation for the treatment of human obesity remains to be developed.

Metabolic bone disease is returning to the limelight of nutritional interest after years of dormancy. Two papers reflect this resurgence. J.A. BENGOA et al from the University of Chicago correlated Serum 25-Hydroxy-vitamin D, Calcium Absorption, and Bone Histology in Chronic Cholestatic Liver Disease patients. Calcium-47 absorption predicted serum 25-OH vitamin D levels, but neither of these two parameters predicted the histological findings in the bones: Two patients had osteoporosis and 2 had osteomalacia in the series of eight cases. (The abstract had nine co-authors!). Several recent papers at these meetings have described, and partially characterized a bone disease that presents in patients on long-term total parenteral nutrition. S.M. OTT from Seattle evaluated quantified bone volumes, tissue area, osteoid, and bone area as well as stainable aluminum in bone biopsies from 16 normocalcemic, normophosphatemic patients on TPN for an average of 37 months (range 8 to 84 months). Vitamin D hormone and parathyroid hormone levels were borderline to low. The dominant lesion was characterized as osteomalacia and aluminum deposition corresponded to the sites of impaired bone mineral deposition. In the patients receiving casein hydrolysates as the parenteral alimentation solutions, substantial contamination with aluminum was encountered. The investigators raised the question of whether, as in their previous experience with patients on hemodialysis, aluminum might not be a factor in the osteomalacia of chronic home TPN.

The program's scientific sessions were rounded out by a number of miscellanous papers on various themes including: Lipoprotein composition in diabetics; amino acid and protein turnover with human studies related to normal adults and patients following surgery, and in relation to acute renal failure and meal-feeding in experimental animals; and immune factors in relation to protein-energy malnutrition in a guinea pig model, and iron status in a rat model. G.E. GAULL and co-workers on Staten Island raised a provocative point in the disucssion of the nutritional essentiality of dietary taurine by demonstrating that Taurine Enhances the Proliferation of Cultured Human Cells. More *in vivo* human work is also required in this field. Finally, J. METCOFF as the head of a collaborative team from Oklahoma, Grand Forks and Beltsville examined a multifactorial, nutritional puzzle relating to the topic Maternal Nutrition Affected by Smoking during Pregnancy. The babies of smoking mothers were indeed smaller, and some nutrients and cell bioactivities in pregnant women were altered. Insufficient dietary data were presented to determine if the static and functional indicators of nutrition were reduced due to a change in dietary intake, or through metabolic mechanisms unrelated to food consumption.

The meetings were well attended, and the participation from the audience was enthusiastic and informed. The quality of presentations and the revelence of the data to clinical nutrition varied widely, as might be expected, and many abstracts of topical interest were not programmed due to constraints on time. These scientific sessions represent a valuable forum for the presentation of new data and for the interchange among nutritionists from hospitals, industry and academia, students and professors, young and old. Their support and development should be the desire of all those involved with the field of clinical nutrition.

NOEL W. SOLOMONS

AMERICAN PEDIATRIC SOCIETY AND THE SOCIETY FOR PEDIATRIC RESEARCH

The combined meetings of the American Pediatric Society and the Society for Pediatric Research were held in Washington May 11-13, 1982. Papers directly related to nutrition were presented in a wide variety of sessions. Although the topics were highly diverse, they will be grouped broadly under the headings "Community Nutrition, Body Composition, Infant Feeding, Breast Feeding and Infection, Growth, and Minerals and Trace Elements". Papers are referenced by chief author and abstract number. Readers who wish further details are referred to Pediatric Research 16:1A-413A, 1982.

Community Nutrition

The decline in low birth weights (LBW) fostered by preventive programs such as WIC and prenatal care will expectedly reduce the incidence of cerebral palsy (CP), in contrast to changes fostering survival of LBW infants, such as neonatal intensive care units (NICU). DAVID et al (421) calculated the impact of these two programs on CP using two studies that provided BW data on all CP cases in a geographic population, vital statistics from North Carolina collected over an 11 year period, and BW and mortality data from the Massachusetts WIC evaluation project. Reductions in the frequency of LBW infants associated with improved prenatal nutrition appear to have compensated for the increased survival attributable to NICUs. Approximately 20-30 new CP cases/100,000 live births appear to be prevented by the WIC program. These data suggest that preservation of federal feeding programs are of considerable importance to the health of the pediatric population.

Body Composition

KLISH et al (538) presented a new method for the assessment of lean body mass in infants. The EMME (electronic meat measuring equipment) operates on the principle that a biological specimen perturbs an electronic field in proportion to its electrical conductivity, which in turn is dependent on its electrolyte content. The correlations of fat and electrolyte concentrations in phantom samples ($r = 0.99$) were linear and highly significant in volumes of 2-6 liters indicating that this method is a promising technique for measuring body composition in infants.

Water labelled with the stable isotope of oxygen, $H_2{}^{18}O$ was used by TROWBRIDGE et al (607) to measure total body water (TBW) in infants. Urine obtained from 5-7 hours after isotope administration demonstrated reliable plateaus of enrichment. However, because extracellular water (ECW) is increased in infants, measurements of TBW must be accompanied by measurements of ECW. Nonetheless, these two techniques allow noninvasive measurements of body composition in infancy.

Infant Feeding

Despite the universal application of nasogastric feeding in premature infants, BERNBAUM et al (1199) reminded us that sucking has functions other than filling the stomach. Transcutaneous O_2 tension was monitored prior to, during, and for 30 minutes after each NG feeding with and without a pacifier. The pO_2 fell significantly during NG feedings, continued to fall for 20 minutes, and returned to baseline levels by 25 minutes after feeding, whereas in NG feeding accompanied by a pacifier, pO_2 rose significantly, stayed

elevated for 20 minutes, and returned to baseline by 25 minutes after the feeding. The improved PO_2 while sucking may represent a physiologic response to meet the increased caloric demands associated with feeding.

Review of the incidence of necrotizing enterocolitis (NEC) over a seven year period indicated an association between the brand of infant formula used in feeding and the incidence of NEC (RICHARDSON et al, 446). Criteria used for the diagnosis of NEC were abdominal distension, hematochezia, and radiographic evidence of pneumatosis intestinalis. In contrast to other years, peak years of occurrence were also associated with NEC in larger babies and an increased frequency of affected babies in the term nursery. During the years in which SMA was used as the standard formula, the incidence of NEC was significantly greater ($p < .001$), whereas the years in which Similac was used, the incidence was significantly lower ($p < .005$) than the years in which Enfamil was used. Because the study was retrospective, no conclusions can be drawn regarding causality; nonetheless, the strong association emphasizes the need for confirmatory data and additional prospective investigations.

The metabolism of specific substrate utilization by infants was examined in several studies. Lactose assumilation was examined in 19 term infants to determine whether breast milk lactose concentrations exceeded the absorbtive capacity of the newborn gut (MACLEAN et al, 548) using ^{13}C-lactose breath tests to measure absorbtion. Lactose absorbtion by the small bowel appears complete; therefore colonic salvage is probably quantitatively less important in term than in premature infants. The utilization of more complex carbohydrates was examined in similar fashion, but relied on the natural enrichment of corn starch with ^{13}C and the appearance of $^{13}CO_2$ as an index of absorbtion and utilization (SHULMAN et al, 593). In a group of one month old infants, 30% of the starch administered appeared to be utilized, as indicated by changes in breath $^{13}CO_2$. Approximately 20% of fecal carbon derived from starch; the absence of stool starch or glucose suggests that colonic flora converted the starch to other non-carbohydrate forms.

Although it has been clear for sometime that the addition of glucose to oral rehydration solutions promotes the absorbtion of sodium, the effect of protein has not previously been examined. Using intestinal profusion in adults infected with Norwalk virus (SMALLEY et al, 594), the effect of Pedialyte versus Pedialyte plus 1% whey protein on jejunal flux of water, chloride, potassium, and glucose was compared. The addition of protein significantly improved the absorbtion of water and electrolytes, but not glucose. These results suggest that the addition of protein to oral rehydration solutions improves jejunal absorbtion of water and electrolytes as well as nutritional quality.

Breast Feeding and Infection

The immunologic effects of breast milk continue to receive the attention they deserve. In an elegant presentation, LUM et al (436) demonstrated convincingly that Alaskan Eskimo infants had a ten-fold higher incidence of severe Hemophilus influenzae Type B (HIB) infections in the first 18 months of life, compared to urban Eskimo, Indian or non-native infants. Using two controls matched by race, village and age for each case, breast feeding had a clearly protective effect against HIB infection, although small numbers of breast fed infants still acquired disease. Anticapsular antibodies levels to HIB were comparable to those in the United States but both IgA and IgM classes in Eskimo breast milk were significantly greater. An unexplained finding was that antibody levels of mothers of breast fed infants with disease were comparable to controls and other mothers of breast fed infants without disease.

According to DWORSKY et al (962),breast milk appears to be a major source of cytomegalovirus (CMV) infection. Eighty-two percent of infants breast fed longer than one month whose mothers were CMV infected at birth became infected. CMV was recovered from breast milk in 31% of the infected mothers studied. Virus was recovered significantly less frequently from the vagina (11%), urine (9%) and saliva (3%). Clearly seropositive women who excrete CMV in breast milk are significantly more prone to infect their infants.

Immunization of breast-feeding and non breast-feeding mothers with attentuated rubella virus (LOSONSKY et al, 886) was followed by nasopharyngeal viral shedding in 30-50% and breast milk shedding in 70-75% of immunized women. Rubella virus was isolated from the nasopharynx of 56% of breast fed infants, followed by a modest antibody and cell mediated immune response in approximately half of the virus positive infants. Virus shedding and seroconversion rarely occurred in nonbreast fed infants. Infection of newborns occurred despite significant concentrations of anti-Rubella IgA in breast milk, regardless of whether the virus was inoculated subcutaneously or intravenously. The magnitude of IgA response corresponded to the quantity of virus excreted. No signs of infection were noted in the infants.

Together, these papers indicate that breast milk may be a significant source for viral acquisition by newborns. In view of the limited immune responsiveness of these infants, either naturally acquired or vaccine infections of nursing mothers should be viewed with caution.

Growth

Our appreciation for the factors influencing intra- and extra-uterine growth rates has increased commensurate with the sophistication of the techniques used to assess this omnipresent concern. A prospective study (FERNHOFF, et al, 1145) of the nutriture of mothers of infants with the fetal alcohol syndrome (FAS) revealed significant inverse correlations between alcohol intake and the infant's gastational age, birth weight, and length. No significant correlations were observed between alcohol intake and maternal height, weight, TSF, hematocrit, serum iron, ferritin, or vitamin A. Maternal plasma and hair zinc were comparable to infants in both controls and drinkers. Therefore maternal nutriture among drinkers appears comparable to controls, and features of the FAS appear quantitatively related to maternal alcohol intake.

Studies of 204 very low birth weight (VLBW) infants (HACK et al, 47) with comparable maternal characteristics suggested that a critical period exists for catch-up growth in small for gestational age (SGA) VLBW infants during infancy. Significant correlates of impaired growth at 21 months were head circumference, social class, marital status, neonatal risk, low developmental quotient, and neurologic abnormality or chronic disease.

Open circuit calorimetry, anthropometry, and a combination of energy and macronutrient balances were used to compare the growth of normally grown (AGA) and SGA VLBW infants (CHESSEX et al, 185). The SGA infants had decreased absorbtion of fat and protein. A hypermetabolic state was documented in these infants and shown to be associated with increased fat oxidation, possibly secondary to an increase in relative brain size. Despite these differences, weight and length gain was comparable, and the energy/gm weight gain lower. These data suggested that an increased proportion of the weight gain was water, but no differences in body water were observed.

The same group (REICHMAN et al, 1353) using the same system, compared the effect of breast milk and formula on growth among these infants. No significant differences in growth rates were observed. Energy expenditure and storage were higher in formula fed infants, but this was a direct function of increased energy intakes. Fat oxidation was greater among breast fed infants and fat accretion was greater among formula fed infants. The direct

relationship between nutrient intake and accretion of energy, fat, and protein suggests that the composition of weight gain can be manipulated directly by changes in energy and macronutrient intake.

A further extension of these studies (CHESSEX et al, 481) showed that own mother's milk was an adequate source of energy and macronutrients to promote growth in premature infants, and that the accretion rates of protein and fat were comparable to those of the fetus of the same age.

The effects of conceptual age and diet on protein metabolism were reported in a small group of preterm infants (NISSIM et al, 562). A bolus injection of ^{15}N-glycine, and the appearance of ^{15}N in urea was used to measure whole body protein metabolism. Three-methylhistidine was used to assess muscle catabolism. Mean rates of whole body protein synthesis and catabolism are 2-5 times higher in premature infants than in young adults. Muscle protein breakdown increases with maturation and contributes a large fraction of flux. The efficiency of protein synthesis/kilocalorie/gm of dietary protein was greatest in young infants and declined with age.

Minerals and Trace Elements

MARKOWITZ et al (14) observed circadian rhythms in blood calcium, ionized calcium, and phosphorus that paralleled those observed in adults. Although the magnitude of these changes was greater, no ontologic change in the pattern was apparent.

Two papers on copper metabolism have significantly enhanced our understanding of the potential genetic and pathophysiologic characteristics of Menkes disease. COX et al (671) used Chinese hamster and mottled mouse somatic cell hybrids, and Southern blot analysis, direct assay and enzyme markers of the hybrid cells to demonstrate that the mouse metallothionein 1 was localized to chromosome 8. Furthermore m-RNA for metallothionein 1 was present indicating that the defect in mice was attributable to an absent or altered regulatory gene or another undefined defect. Studies of cultured skin fibroblasts from the same animal (PACKMAN et al, 694) showed that copper, zinc and cadmium accumulation were comparable but, in contrast to copper, efflux of zinc and cadmium were not impaired. In control fibroblasts copper accretion increased during coincubation with zinc or cadmium but did not change when mottled mouse fibroblasts were coincubated with the same elements. Therefore, the defect in the mottled mouse appears to affect the function of a copper transport or storage protein, but metabolism of zinc and cadmium is not affected. The absence of induction of the transport protein in response to zinc or cadmium may reflect altered function or regulation of the copper transport or storage protein.

WILLIAM H. DIETZ, JR., M.D., PH.D.

CANADIAN FEDERATION OF BIOLOGICAL SOCIETIES

The conference was held in Edmonton, June 14-18, 1982. The first day program was highlighted by the 25th Anniversary Symposium of the Canadian Society for Nutritional Sciences on the futurist theme of The Next Twenty-Five Years: New Challenges for Nutritionists. WALKER discussed the metabolic aspects of lipids, summarizing the new knowledge bearing on the relationship between fats and prostaglandins, leukotraenes and other mediators. The role of dietary factors, particularly salt and protein, in the genesis of hypertension was reviewed by MOGENSON, much of the work being based on studies in the hypertensive rat model. ANDERSON discussed the relationship between dietary amino acids and brain neurotransmitters and how both short-term and long-term changes in food intake might affect brain chemistry and function. BROWN decsribed elegant studies on the role of food and gastrointestinal hormones particularly GIP. BRUCE summarized the current information on the role of dietary factors, nutrients as well as toxicants, in the production of cancer in man and animals. He indicated that fecal analysis may be an important clue to the presence of chemicals that are important in the production of colon cancer. CHANDRA reviewed the relationship between nutrition and the immune system and indicated that investigations in this field have progressed from historical descriptions to phenomenology, mechanisms, role of single nutrients, immunoregulation and the utilization of available information in the management and prevention of malnutrition-infection comlex. BASU outlined the various interphases between nutrition and drug metabolism. The symposium proceedings will be published shortly.

FLANAGAN showed that zinc deprivation lowers endogenous zinc excretion in mice and encourages its active retention by the proximal intestine. ANDERSON compared hair levels of trace elements in two groups of infants from Halifax and Guelph, and found higher levels of zinc in the former who were also heavier and taller. The differences in hair zinc were attributed to water hardness, the high concentration of calcium in Guelph water may have decreased intestinal zinc absorption. LEUNG estimated the sodium intake of infants up to 18 months of age by the 4-day dietary record method. The mean sodium intake was substantially higher than RDI and from 8 months onwards it was 300% RDI. COLE described the metabolic basis of hypercalciuria and osteopenic bone disease in patients maintained on prolonged TPN. It was found that serum suphate and renal sulphate excretion were increased. Addition of cysteine to the infusate provoked a further increase in serum sulphate and urinary sulphate. AMIOT studied selenium absorption and retention in relation to soya bean protein intake and found that selenium homeostasis is maintained largely through changes in urinary excretion of the trace element and that inadequate protein diet reduces the efficiency of retention. ATKINSON examined the relationship between renal functional changes and renal chemical and microscopic calcium in piglets receiving oral 25-hydroxyvitamin D3 and found less than 5 ug/kg/day to be an optimal dose. Overdosage resulted in diminished renal function even in the absence of hypercalcemia or calcium accumulation in the kidney. Species difference in vitamin D toxicity was apparent from the paper by HILTON who demonstrated that trout are resistant to excess vitamin D3 and such high doses are not associated with increased incidence of renal calcinosis.

NINA investigated the effect on plasma cholesterol of replacing the polyunsaturated fat in an infant formula by a more saturated fat to simulate breast milk more closely. The plasma cholesterol level of the breast fed infants was 45% higher at two months and 20% higher at six months than those of the formula-fed infants irrespective of the type and amount of fat. ZLOTKIN looked at neutrophil function in vitro and its relationship to concentration of lipid emulsions. Concentrations above 100 mg% were associated with reduced bactericidal capacity. KOSTOLANSKY found that animals raised in large litters begin to consume solid food earlier than those raised in litters of 9 or 3. Animals in larger litters showed elevated activities of G-6-PD, malic enzyme and citrate cleavage enzyme. WILK-GEROW found that enzyme activity in the liver was generally independent of dietary fat but sex and age effects were apparent. ANDERSON described a probability approach to estimating nutrient deficiencies in a population from dietary records. First, mean nutrient intakes were compared to RDNI. Second, the proportion of the population not meeting the RDNI was calculated . Third, the distribution of nutrient intakes below the RDNI was determined and combined with probability statistics to calculate the number of subjects likely to have intakes below their own individual requirements. The first approach underestimated the possible problems whereas the second approach overestimated them. The third method appeared to predict nutrient deficiency incidence which was more in agreement with biochemical evidence.

BHULLAR found the glycine cleavage activity to be 10 times higher in the kidney as compared to liver of normal rats. In experimental biotin deficiency, the liver enzyme activity did not change whereas the kidney enzyme was 25% of normal. In a poster presentation, SRIVASTAVA indicated that continuous undernutrition in five successive generations of rats produced reduction in body and organ weights, nucleic acid and protein concentrations of various organs. Total nucleotide and leucine content did not vary. TUCHWEBER compared the activities of four lysosomal enzymes in whole liver homogenates and in suspension of isolated parenchymal cells during aging of female rats, fed ad libitum or subjected to graded dietary restriction until 21 months of age. Between 6 and 21 months of age, the specific activities of arylsulphatase B and cathepsin D increased whereas that of acid phosphatase and b-galactosidase decreased.

DESAI provided dietary supplements to agricultural migrant workers in Brazil and found that physical work capacity as measured by heart rate in response to different work loads on a cycloergometer improved. JOHNSTON measured catecholamine metabolism in obese adult females. The obese excreted less urinary metabolites of norepinephrine and did not respond to tyrosine. JACQUES measured neurotransmitter synthesis in rats, rabbits and cats fed casein and soy protein. In soy-fed animals, a greater 5-HT utilization and catecholamine mobilization was observed.

These and many other presentations indicated that high quality research in nutritional sciences continues to be carried out in Canadian institutions.

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REVIEWS OF BOOKS

Infections of the Gastrointestinal Tract

Herbert L. DuPont and Larry K. Pickering. Plenum Medical, New York. pp 273, 1980. Price $24.50.

This is the second volume in the series Current Topics in Infectious Disease. Infectious diarrhoea continues to be a major cause of morbidity and mortality worldwide. It contributes to malnutrition and its control is an important objective of most preventive programs. This excellent book describes the syndromes produced by specific agents of diarrhoea, including amebiasis, giardiasis, bacillary dysentery, salmonellosis, cholera, E. coli, viral causes and food-borne diarrhoeal illness. There are good chapters on diagnosis, treatment and fluid therapy. The opening chapter on Immune Mechanisms is the weakest which may reflect the authors' own background. One would have liked to see description of newer vaccines for the control of diarrhoea. In spite of these deficiencies, the book brings together a wealth of useful information.

Essays on Pediatric Nutrition

David L. Yeung, Editor. Canadian Public Health Association, Ottawa. pp 199, 1981. Price 10.00.

This is a compedium of reviews on a variety of topics related to nutrition of infants and children. Many were based on presentations made during the Year of the Child Symposium cosponsored by the Nutrition Society of Canada, American Institute of Nutrition and American Society for Clinical Nutrition. JACK METCOFF provides an excellent analysis of maternal nutrition and fetal development, whereas GRAHAM CHANCE describes the progress and problems in the field of nutrition of the low birth weight infants. KATHRYN BARNARD addresses general issues in parent-infant interaction during the first years of life. MINA McDANIEL summarizes the sensory evaluation of infant foods. RANJIT CHANDRA provides an overview of the interactions between nutrient intake, immunocompetence and susceptibility to infectious illness. DAVID YEUNG discusses infant obesity and principles of infant feeding. STEPHANIE ATKINSON describes factors affecting breast milk composition. The sections on Breast Milk, and Atherosclerosis Risk Factors are very sketchy and superficial. In general, the essays provide a good state-of-art information on highly topical themes.

Health and Disease in Farm Animals

W.H. Parker. Pergamon, New York. 1980, pp 307. 3rd edition.

A new edition of a concise good text on the subject.

Nutrition and Nutritional Diseases: The Evolution of Concepts

Karl Y. Guggenheim. Collamore Press, Lexington, Massachusetts. pp 379, 1981. Prince $27.50.

In teaching nutritional sciences, we are apt to forget the evolutionary process that preceded modern concepts and knowledge. Most texts neglet this important historical perspective. This volume describes the historical development of basic concepts in nutritional science, neginning with Greco-Roman antiquity and and leading to present theories. The author describes some crucial experiments and observations and provides biographic summaries of the scientists involved in the advancement of nutritional thought and knowledge. The main deficiency of the book lies in its neglect of the contributions made by scholars in other lands and eras, particularly the Chinese and Indian sages and observors. There is paucity of illustrations which would have enlivened the text and made it more enjoyable and palatable to digest. Nevertheless, the book makes interesting reading.

The Science of Food

P.M. Gaman and K.B. Sherrington. pp 245, 1981. Second Edition. Pergamon Press, New York.

The book is intended primarily for catering students and provides a useful elementary text on the topic.

Textbook of Gastroenterology and Nutrition in Infancy

Emanuel Lebenthal, Editor. Raven Press, New York. pp 1133 in two volumes, 1981. Prince $98.00.

This large text provides an overview of the basic science and clinical aspects of the interrelated topics of nutrition and gastroenterology as applied to young children. The normal digestive processes and biochemistry are described followed by pathological states. The various sections include intrauterine nutrition, growth and development, ontogeny of the gastrointestinal tract, development during the neonatal period, immunologic and bacteriologic aspects, nutritional requirements, human breast milk, protein, carbohydrates, fats, vitamins and trace elements in infant nutrition, adverse effects of current feeding practices, protein-calorie malnutrition, nutritional deficiencies, gastro-intestinal disorders of infancy, including chapters on hepato-splenomegaly and cholestasis. The publication appears to be based on a symposium proceedings and there are obvious overlaps and omissions. The quality of various chapters differs enormously. Most are average to poor; a few are excellent. A stronger editorial direction and control would have improved the quality of the publication.

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EDITORIAL

Nutritional Assessment of the Individual

The importance of dietary factors in the pathogenesis of disease and its complications has stimulated studies evaluating the nutritional status of individuals and populations. Dietary intake data, physical examination, biochemical analyses and anthropometry are the main methods used for this purpose. Recently, investigators have looked at various functional indices of nutritional deficiency, including immunocompetence (1), work performance (2) and cognitive function (3). Moreover, new methodologic advances in stable isotope technology have permitted the use of safer materials and more sensitive and specific assays (4). Comprehensive reviews provide recent information on the subject of nutritional assessment (5-7).

There are numerous difficulties in the correct estimation of an individual's nutritional status. Dietary intake data, for 1-3 days, even though assumed to be an accurate reflection of long-term food intake, cannot be directly translated into nutritional status because factors of digestion, absorption, metabolism, and excretion, can influence it. The problems are compounded when dealing with certain age groups, particularly the elderly. In those over the age of 65 years, many of the conventional tests of nutrition have little validity. For those who are ill, assessment must reflect the effects of specific disease states. The interactions between various tests of nutritional status, viz. anthropometry, biochemistry and body composition, are of fundamental importance. Finally, it is being recognized that there are static 'standards' of limited use and that we should define thresholds that separate normal function from dysfunction.

Serum thyroxine-binding prealbumin concentration has been suggested to be a sensitive indicator of protein-energy status of individuals. It has a short half-life of about two days. Its levels fall rapidly following dietary restriction and rise quickly after supplementation. The need for a reliable method for determining sub-optimal nutritional state in patients with chronic intestinal disease led to the studies reported by Farthing in this issue of the Journal. He concluded that serum thyroxine-binding prealbumin is unreliable for nutritional assessment in such patients and that rapid changes in its concentration may mask undernutrition.

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NUTRITION AND HEALTH OF THE RURAL ELDERLY IN FIVE SELECTED BARANGAYS IN THE PHILIPPINES[1]

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F. J. Serrano[4], B.S., and A. G. Tan[4], M.D., M.P.H.

ABSTRACT

A group of 100 individuals (55 women; 45 men) aged 60 years and over participated in the study. They were residents of five selected rural communities or barangays in Laguna and Batangas (southern Tagalog region). Data obtained included some background information, dietary intake based on a 24-hour recall, physical assessment with health history and limitation of activity status, and selected anthropometric measurements. Mean energy intakes correlated directly and significantly with protein, carbohydrate, fat, calcium, iron, thiamin, riboflavin, and preformed niacin but not with vitamin A value and ascorbic acid. Mean intakes by age groups met (a) less than 65 percent of the local recommended dietary allowances for Filipinos for vitamin A value and ascorbic acid and (b) 65 percent or more of the allowances for protein, iron, thiamin, and preformed niacin. About half the rural elderly had health ratings of "good," and a third rated "fair." Nearly 40 percent had average body weights, and more than 50 percent were underweight by local standards. The low body weights for height reflect the small amounts of subcutaneous fat (based on skinfold thickness) and muscle tissue (based on arm and calf circumferences). Body weights correlated significantly and positively with triceps and subscapular skinfolds and with arm and calf circumferences. Body weights did not correlate significantly with food energy intakes.

KEYWORDS: nutritional health, rural elderly, Philippines

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INTRODUCTION

There is growing interest in and concern for the health and welfare of the elderly in developing countries. Population predictions indicate a dramatic rise in the proportion of older people in these regions by the year 2000. To create and raise international awareness of these aging trends, basic information is needed on the aging and the aged in developing nations (1).

People aged 65 and over make up less than 4 percent of the total population in "young" countries such as the Philippines (2). But numerous and complex forces are operating in many developing societies that may change conditions fast and drastically. In the Philippines, for example, successful implementation of measures to reduce infant and child mortality, and to control population growth undoubtedly will change the age composition of the population. Older people will increase both in number and proportion. In addition, as developing countries become industrialized and income per capita increases, the traditional family support system of the elderly may be disrupted. These predicted trends may have profound and far-reaching effects on the family and, eventually, on the entire society. A sense of national and international commitment is urgently needed to help young nations cope with these predicted challenges. The 1982 World Assembly on the Elderly (3) will focus on aging individuals and societies both in developing and developed nations.

The present study was conducted to explore some aspects of nutrition and health of the rural elderly in five selected barangays in the Philippines. A "barangay" is the smallest political unit designated by a Presidential Decree in September 1974 (4). Nearly 70 percent of the total population in the Philippines is rural, and its economy is predominantly of subsistence nature. Many of the rural families are poor and large, and literacy level is low (5). The five-year (1978-1982) nutrition plan for the Philippines emphasizes planning at the grassroots or barangay level and "percolating up" to the regional and national levels (6).

METHODS

Background Information

The present study was integrated into an ongoing local project entitled "Food Self-Sufficiency Program for the Nutritional Improvement of the Rural Poor." This model program was initiated in May 1978 by the Institute of Human Ecology and the University Extension Center of the University of the Philippines in Los Baños (UPLB), Laguna, Philippines. Six barangays were selected in the two adjoining provinces of Laguna (3 barangays) and Batangas (3 barangays). They were identified for the program on the basis of (a) availability of qualified village workers who can be trained to carry on the program activities under a field coordinator and (b) diversified food and crop production representative of the rural Philippines. The food and crops produced in these project sites are rice, sugarcane, coconut, corn, vegetables, and fish.

For the study of the rural elderly, five of the six barangays were included: the sixth community, a fishing village, was inaccessible for nearly 3 months after a severe typhoon during the latter part of October 1978.

Before our field observations of the elderly, we (PAG, TL, FJS) visited the rural health units that serve the barangays. The medical officer and (or) other local health staff oriented us to the health service delivery system for each rural community. We, in turn, informed them about our study of the elderly and solicited their assistance. All the health units expressed willingness to cooperate and support our study. Barangay officials and workers also were contacted and informed about our study.

Subjects

A sample of 100 individuals (55 women and 45 men) aged 60 years and over was drawn from relatively homogeneous rural populations of the five study barangays. On the basis of previous nutrition survey reports and estimated proportion of the 60-and-older group (about 4 percent), an arbitrary sample size of 20 individuals per barangay was considered appropriate for this study over a limited time. All residents 80 years and older of a given barangay and willing to cooperate were included in the sample. The balance of the group was randomly selected from the 60 to 79 age bracket. Sixteen percent of the entire group of 100 individuals were not available at the scheduled visits, and they were replaced by randomly selected alternates.

Form letters in Pilipino (national language) were sent in advance to each individual chosen, to explain briefly and clearly the nature of our study; the date, time, and place of our visits to the barangays were included. The barangay officials and workers reinforced our recruitment efforts. On the day of our visit, the local workers contacted the elderly volunteers and directed them to our site for interview and measurements. Cooperation was excellent.

Data Collection

A trial run preceded the actual data collection. The study period of all five barangays extended from October 24 through November 29, 1978. Observations included dietary interviews, physical (health) assessment, and measurements of selected body dimensions. Three local research assistants conducted the interviews in the native language. One interviewer took dietary information based on a 24-hour recall. Pictorial food models and local utensils and objects were used to help estimate amounts of foods and beverages consumed; the other two interviewers obtained the health histories and served as interpreters for the public health nurse in the study team. These three research assistants were recent graduates of the UPLB with B.S. degrees in Home Technology, major in Foods and Nutrition. One of the three assistants (FJS) took the research option, and she served as our senior research assistant and liaison. The principal investigator (PAG) had a working knowledge of the local language and was familiar with the regional culture. The public health nurse (TL) from the U.S.A. did the physical assessment. Selected body measurements were taken by the nutritionist (PAG). The sites for the field visits included (a) the homes of the barangay officials for barangays 1 and 2, (b) the home economics classrooms in the elementary schools for barangays 3 and 4, and (c) the local chapel for barangay 5.

The interview schedule and record forms were prepared in the U.S.A. for field use in the Philippines. As expected, some modifications had to be made to fit local conditions. Coding guidelines and scoring systems were developed (by TL and FJS) for the physical assessment and limitation of activity status. The public health nurse and the senior research assistant worked together on this particular phase throughout the study with the guidance of our medical consultant (AGT).

The indicators of health status were based on the (a) health score from 47 items in the physical assessment schedule and (b) limitation of activity status score from five items in a separate schedule. The physical assessment included a health history and regional review (general; skin, hair, nails, head, eyes, ears, nose and throat; neck; cardiorespiratory; gastrointestinal; genitourinary). Measurements of respiratory rate, pulse rate, and blood pressure were taken. Blood pressure was measured with a Cardy-8-Mini Electronic Blood Pressure Meter (Burlington Bio-Medical Corporation, Plainview, New York).

Information on limitation of activity was an attempt to measure long-term disability due to chronic conditions (7). In the absence of role or major activity designations for the elderly, the categories used were "housewives" and "workers." After the major activity was identified, the individual was classified according to one of three categories, ranging from no limitation in activity to complete inability to carry on the major activity. The rating scale used for each item in the health and limitation of activity status schedules consisted of scores from 1.0 to 5.0 with 1.0 = Poor, 3.0 = Fair, and 5.0 = Good. A weighting system was employed for the final health score, with 60 percent assigned to significant diseases reported by each individual and 40 percent for the remaining items. For statistical purposes, these scores were grouped into three intervals: 1.0-2.3, 2.4-3.6, and 3.7-5.0. Because the health score and the activity score were significantly and directly correlated with each other ($r = 0.31$, $P \leq 0.05$), the two scores were combined, averaged, and used in subsequent analyses.

Selected body measurements were made according to standard anthropometric techniques (8) with some modifications on the tools employed. The individual was weighed with ordinary light clothing (heavy objects in pockets were removed) and without shoes or slippers. A new spring, automatic indicating portable scale (capacity 100 kg. with 0.5 kg. graduation; Yamato, Japan) was used. Calibration by the local Test and Standards Laboratory indicated an error of ± 0.1 kg. for readings between 20 and 80 kg. Body weight status was based on percentage deviation from the recommended body weight. The recommended body weight was estimated by following Thannhauser's method (9):

Height in cm minus 100 = Recommended weight (Rwt) in kg.

Rwt minus (Rwt x 10%) = Recommended weight adjusted for the smaller body frame of Filipino adult.

Another approach used consisted of predicting recommended weight for a given height from regression equations based on data for adult Filipinos (9; Table 3 in text). Height was measured with two wooden meter sticks placed end to end and securely attached with masking tape to the wall (straight surface with no baseboard). Sitting height was taken in the same manner as the standing height; it is the distance from the horizontal sitting surface to the top of the head. Circumferences of the upper arm and calf were measured with a flexible metal tape (1 m long, 6.5 mm wide and graduation in cm). Skinfold thickness was taken at four sites: triceps, left and right arm, subscapular and supra-iliac, right side. A Lange skinfold caliper (Cambridge Scientific Industries, Cambridge, Maryland) was used. Grip strengths of the right and left hands were recorded by the use of a hand dynamometer (Model 78010, 0-100 kg; LaFayette Instrument Co., LaFayette, Indiana).

Data Coding and Analysis

Data were coded for subsequent computer processing and analysis. The nutritive value of the diet was calculated by the use of the local food composition table (10). Dietary adequacy was evaluated by comparison with the 1977 Recommended Dietary Allowances for Filipinos (11). Statistical analysis was based on the Statistical Analysis Systems[1] (SAS) computer programs for analysis of variance and Duncan's Multiple Range Test. Correlation coefficients also were calculated. The statistical probability of 0.05 was considered significant.

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[1]Barr, A. J., Goodnight, J. H., Sall, J. P., and Helwig, J. T. SAS Institute Inc., P.O. Box 10066, Raleigh, N.C. 27605.

RESULTS

Profile of the Rural Elderly

Some characteristics of the rural elderly in the samples are shown in Table 1. Females predominated over males in number for the entire sample and for four of the five barangays. For the total sample of 55 females and 45 males, the sex ratio of 82 indicates 82 males for every 100 females. Similar trends have been reported for the world and its major regions (12).

Ages ranged from 60 to 98 years for the women and from 59 to 91 years for the men. One male aged 59 years was inadvertently included in the sample. The oldest woman, who claimed to be 98 years old, did not know her exact date of birth. Two-thirds of the total sample were in the 60-69 age group, with 25 percent in the 70-79 age bracket. The remaining 8 percent were 80 years and older.

More than half the group were married, and there were more widows than widowers. In many parts of the world, women tend to outlive men (12, 13). Life expectancy at birth is greater for females than for males. According to the 1975 Census, the life expectancy at birth in the Philippines is 61.9 years for females and 56.2 years for males. These sex trends are predicted to continue to the year 2000.

When interviewed, nearly half of the group was engaged in farming, with more men than women. A third did housekeeping, and all were women. About 20 percent had retired.

The majority (80%) had 6 years or less of formal education, and 15 percent did not attend school. Nearly all the people interviewed rated "good" in ability to communicate and comprehend in Pilipino.

All but one individual came from the Tagalog region, which includes Southern and Central Luzon provinces. Therefore, one would expect more cultural similarities than differences among the elderly participants. Roman Catholics predominated over non-Catholics. Fifty-eight percent had lived more than 50 years in their place of residence at the time of the study.

Some aspirations expressed by the rural elderly of this study reflect the worldwide attitudes toward longevity (14). Most of the people, especially those in their 60's and 70's, want a long and comfortable life. To over a third of the group, a "good life" implies a comfortable life; to another third, a sense of accomplishment makes a good life. Eating three meals a day is a "good life" to 19 percent, and only 10 percent aspired for "good health condition."

Nutritive Value of the Diets

The mean dietary intakes of the elderly females and males were based on one 24-hour recall record per individual. Table 2 gives the nutritive value of the reported diets by age groups. Because of the small sample size for ages 80 years and older, age effects were examined on the basis of two age categories: ages equal to or less than 69 years and ages greater than 69 years. In the analysis of variance, mean values are adjusted for identified sources of variation included in the model used. For the Duncan's Multiple Range Test, the unadjusted means or original data (as shown in Table 2) are used. The results from these two analyses may or may not agree. One, however, would expect the adjusted mean values to give a better estimate of statistical trends than do the unadjusted values. The sources of variation examined were barangay, age, combined health and activity limitation score, and interactions among these three factors.

TABLE 1
Some Characteristics of the Rural Elderly

Characteristics	Distribution of individuals			Characteristics	Distribution of individuals		
	Female	Male	Total		Female	Male	Total
Number of individuals	55	45	100	Religion			
Sex ratio[a]	82			Roman Catholic	50	40	90
Age groups, years				Non-Catholic	5	5	10
59 - 69[b]	37	30	67	Regional Origin[c]			
70 - 79	13	12	25	Tagalog	55	44	99
80 - 89	3	2	5	Bicolano	0	1	1
90 and over	2	1	3	Ability to communicate and understand[d]			
Marital status							
Married	23	33	56	Good	52	43	95
Widowed	32	11	43	Average	3	0	3
Single, never married	0	1	1	Fair	0	2	2
Occupation, present							
Farmer	17	29	46	Length of residence in current barangay, years			
Housekeeper	31	0	31				
Retired	5	13	18	Less than 10	3	1	4
Others[e]	2	3	5	10 - 25	10	8	18
Education[f]				26 - 50	9	11	20
6 years or less	46	34	80	51 - 75	28	23	51
10 years or less	1	3	4	76 and over	5	2	7
More than 10 years	0	1	1				
None	8	7	15				

[a]The number of males for every 100 females.

[b]One of the male subjects was 59 years old.

[c]Based on the dialect used in the region: Tagalog, the basis of the national language, is native to the provinces in southern and central Luzon region; Bicolano is the native dialect of the Bicol region in Southeastern Luzon. Cultural differences between regions exist to varying degrees.

[d]The last category of "poor" had no representation.

[e]Includes three categories: vendor, quack doctor, laborer.

[f]Years of school attendance designated: elementary, high school, and post-secondary education.

TABLE 2

Nutritive Value of the Daily Diet of the Rural Elderly

	Mean intakes ± S.D.[a]			RDA[b]	
	Age groups, years			Age groups, years	
	60 - 69	70 and over	Total	60 - 69	70 and over
A. Females n	37	18	55		
Food energy, kcal[c]	1500 ± 650	1400 ± 550	1450 ± 600	1540	1340
Protein, gm	49 ± 28	41 ± 20	46 ± 26	54	54
Fat, gm	13 ± 13	10 ± 8	12 ± 12	-	-
Carbohydrate, gm	289 ± 127	274 ± 115	284 ± 123	_	_
Calcium, mg	439 ± 374	301 ± 198	394 ± 332	500	500
Iron, mg	12 ± 9	9 ± 4	11 ± 8	8	8
Thiamin, mg	0.7 ± 0.5	0.5 ± 0.7	0.6 ± 0.6	0.8	0.7
Riboflavin, mg	0.5 ± 0.3	0.4 ± 0.2	0.5 ± 0.2	0.8	0.7
Niacin, mg	13 ± 8	12 ± 7	12 ± 7	13[d]	13[d]
Vitamin A, IU	1661 ± 2297	1595 ± 1827	1639 ± 2138	3800	3800
Ascorbic acid, mg	32 ± 34	24 ± 36	29 ± 35	70	70
B. Males n	30	15	45		
Food energy, kcal[c]	2300 ± 1200	1750 ± 600	2100 ± 1050	2060	1810
Protein, gm	70 ± 38	54 ± 26	65 ± 35	63	63
Fat, gm	19 ± 22	6 ± 4	15 ± 19	-	-
Carbohydrate, gm	443 ± 225	358 ± 125	415 ± 200	-	-
Calcium, mg	434 ± 179	507 ± 321	458 ± 235	500	500
Iron, mg	16 ± 8	15 ± 9	15 ± 8	10	10
Thiamin, mg	0.9 ± 0.6	0.7 ± 0.5	0.8 ± 0.6	1.0	0.9
Riboflavin, mg	0.7 ± 0.4	0.6 ± 0.3	0.7 ± 0.4	1.0	0.9
Niacin, mg	19 ± 11	14 ± 8	17 ± 10	14[d]	13[d]
Vitamin A, IU	2693 ± 3177	2324 ± 3688	2570 ± 3318	4500	4500
Ascorbic acid, mg	48 ± 41	45 ± 45	48 ± 42	75	75

[a]Unadjusted mean intake and standard deviation.

[b]Recommended Dietary Allowances for Filipinos per day for specific nutrients. Food and Nutrition Research Institute Publ. 75, Revised May 1977. Body weights: reference woman 48 kg, reference man 56 kg.

[c]Mean values for food energy were rounded to the nearest 50 kcal.

[d]Expressed in mg equivalents.

For the females, barangay effects were significant ($P \leq 0.05$) with food energy, protein, fat, and carbohydrate but not with the other seven nutrients examined. The unadjusted mean intakes of food energy for the five barangays, respectively, were (rounded to the nearest 50 kcal): 1600, n = 13; 1450, n = 11; 1100, n = 12, 1650, n = 7, 1550, n = 12. Age and the combined health and activity limitation scores had no significant effects on the mean dietary intakes.

In the males, barangay did not affect significantly the mean intakes of food energy and 10 nutrients. Age effects were significant ($P \leq 0.05$) on riboflavin and ascorbic acid. The combined health and activity limitation scores had no significant effects on the nutritive value of the diets.

The mean intakes by age groups for the elderly women and men were compared with the local Recommended Dietary Allowances (RDA) for Filipinos (11). For both age groups of females and males, the mean intakes of food energy approached or exceeded the RDA but standard deviations were large (Table 2). Two-thirds or more of the allowances for protein, iron, thiamin, and preformed niacin were met by the group mean intakes of the women and men. The men also had mean intakes of riboflavin and calcium that exceeded two-thirds of the recommended amounts. For the women, group mean intakes of riboflavin failed to meet two-thirds of the allowances; the calcium intake of the older group met less than two-thirds of the local RDA but the younger group exceeded 65 percent of the calcium allowance. The mean ascorbic acid and vitamin A value of the diets of women in the two age groups were below 50 percent of the local RDA. For the men, group mean intakes of vitamin A and ascorbic acid did not meet two-thirds of the recommended amounts. The mean intakes of several nutrients by both women and men varied greatly as indicated by the large standard deviations particularly for calcium, ascorbic acid, and vitamin A (Table 2).

Food energy was positively and significantly correlated with all nutrients examined except for ascorbic acid in the females, and for ascorbic acid and vitamin A in the males. With the exceptions of ascorbic acid, vitamin A, and fat, the remaining seven nutrients were significantly correlated with each other.

The basic diet of the rural elderly in this study resembled the pattern reported in a nutrition survey of the Southern Tagalog Region (15). It consisted essentially of rice, fish, some vegetables and/or fruits. The distribution of calories among the three energy-yielding nutrients was as follows:

	Mean distribution of calories, %		
	Protein	Carbohydrate	Fat
Females	12	80	7
Males	13	81	4
Southern Tagalog Region (15)	10	78	11

In our study, the calorie contributions from protein and carbohydrate were higher, and fat lower, than earlier data obtained from the regional survey. Possible sources of discrepancy include differences in (a) dietary methodology used and (b) sample population observed. In the present study, food records were based on a single 24-hour recall. The regional survey used a 3-day weighed food inventory. In this study, observations were obtained on a limited sample size of a specified age group, the 60-and-over individuals in selected sites. The regional dietary survey, on the other hand, covered 368 households with 2,502 members of widely varying ages.

TABLE 3

Body Weight Status of the Rural Elderly

Classification	Deviation from recommended weight[c]	Distribution of individuals					
		Thannhauser method[a]			Predicted method[b]		
		Female	Male	Total	Female	Male	Total
Average	± 10%	20	19	39	19	20	39
Underweight	- 11% and below	25	21	46	33	20	53
Overweight	+ 10 to 20%	9	3	12	3	3	6
Obese	+ 21% and above	1	2	3	0	2	2
	Total	55	45	100	55	45	100

[a]Recommended weight based on the following:

Height in cm minus 100 = recommended weight (Rwt) in kg.

Rwt - (Rwt x 10%) = recommended weight adjusted for the Filipino adult.

[b]Recommended weight for height based on the following prediction equations (de Leon et al., 1975. Phil. J. Nutr. 28: 1-6).

For females Y (kg) = -52.2649 + 0.6591X (cm)

For males Y (kg) = -71.5026 + 0.7833X (cm)

[c]Obtained by: $\frac{\text{Difference between actual and recommended weight}}{\text{Recommended weight}} \times 100.$

Trends in meal patterns of the elderly participants also were examined. The noon and evening meals supplied the largest portion of the day's food energy intake (35 and 32 percent, respectively). Only about half the group (55 percent) reported eating between meals, and 12 percent of the food energy intake was derived from these snacks. As expected, increasing proportions of food energy from the meals and snacks were associated with a concomitant rise in the percentages of nutrients supplied daily.

Body Weight Status and Health Index

Table 3 shows the body weight status expressed as percentage deviation from the recommended weight. On the basis of appropriate weights estimated by the Thannhauser formula for the entire group, 39 percent had average weights, 46 percent were underweight, 12 percent were overweight, and 3 percent were obese. When recommended weights were calculated by the use of the prediction regression equations for Filipino adults, percentage deviations of body weights were distributed as follows: average = 39, underweight = 53, overweight = 6, and obese = 2. Therefore, the Thannhauser method tends to underestimate the number of underweight individuals and to overestimate the number of overweight individuals. Comparison of the individual recommended weights derived by the Thannhauser formula and predicted by the regression equations showed close agreement when heights exceeded 145 cm, but not for stature below 145 cm. Consequently, the 45 males in the sample with heights ranging from 150.2-171.1 cm showed better agreement in ideal weights based on the two methods than did the females whose heights ranged from 129.5-156.5 cm.

When the health scores and limitation of activity scores were combined and averaged, the men and women showed the same mean score of 3.5. Based on the three scoring intervals used, a score of 3.5 falls in the upper limit of "Fair" (range 2.4-3.6). Examination of individual combined scores indicated that 53 percent of the rural elderly in this study had ratings in the 3.7-5.0 category and that 35 percent were in the 2.4-3.6 interval (Table 4). More than half the females and males in the 60-69 age bracket had mean scores in the upper range (3.7-5.0), and less than half of the older group were in the same upper category.

TABLE 4

Combined Health and Limitation of Activity Scores of the Rural Elderly

Combined score category[a]	Distribution of Scores								Total
	60-69 years				70 years and over				
	Female		Male		Female		Male		
	n	%	n	%	n	%	n	%	n
A. 1.0-2.3	2	5	4	13	3	17	3	20	12
B. 2.4-3.6	12	32	8	27	9	50	6	40	35
C. 3.7-5.0	23	62	18	60	6	33	6	40	53
Total	37		30		18		15		100

[a]The three categories represent the following designations: A = Poor, B = Fair, C = Good.

In the nutrition survey of the southern Tagalog region (15), clinical observations reported for 109 men and women aged 60 and above indicated "fair" ratings for "general appearance" of the majority. In the present study, the main health complaints elicited from the elderly participants included a high proportion of "blurred vision" (65 percent) and "arthritic discomfort" (54 percent). Other conditions indicated were hypertension, 28 percent; gastro-intestinal disorders, 16 percent; "weak lungs" or tuberculosis, 13 percent; cataracts, 9 percent; and enlarged thyroid glands, 7 percent (women only). This information reported by the individuals may or may not reflect accurately the medical diagnoses; more than half the group (62 percent) had indicated recent visits within the past year to a medical doctor. Only 6 percent claimed that they had "never" visited a doctor. Vital statistics for the Philippines in 1974 showed that tuberculosis (respiratory) ranks first as the leading cause of death among individuals 65 and older; heart disease and pneumonia rank second and third, respectively (16).

The combined health and activity limitation score correlated significantly ($r = 0.27$; $P \leq 0.05$) with body weight status for the females but not for the males. Deviations from recommended weights based on the Thannhauser formula ranged from -32 to +20 percent for females and from -35 to ±26 percent for males. The combined score did not correlate significantly with body weight per se for either sex.

Anthropometric Measurements

Selected anthropometric and skinfold thickness measurements by age groups are given in Table 5. Mean values for each of all four skinfold sites measured in females exceeded corresponding values for the males. These same general trends have been reported for other population groups in the world. Most of the elderly participants were right-handed, which, in turn, reflected greater grip strength for the right compared with the left hand. In both men and women, the barangay effects were not significant for most of the body measurements taken.

Age had significant effects on several variables in the elderly females, namely, body weight, arm and calf circumferences, all four skinfold thickness measurements, and left-hand grip. Women less than 70 years had higher unadjusted mean values for these measurements than women 70 years and older. For the males, grip strengths of both hands showed significant age effects ($P \leq 0.01$) with the older group having lower mean values than the younger group.

For the elderly females but not for the males, the health and activity limitation (HAL) scores had significant effects ($P \leq 0.05$) on the grip strength of both hands. High grip strengths were associated with corresponding high HAL ratings (range 3.7-5.0) among the women. In the elderly men observed, age rather than HAL scores determined hand grip strength. A decline in hand grip probably reflects the decrease in farming activities as age increases; generally, both hands are used in this occupation. For people who are conditioned to manual labor such as farming, hand grip may not be a satisfactory index of muscle strength.

All the body measurements taken correlated directly and significantly with body weight except for the supra-iliac skinfold in males. The supra-iliac skinfold was difficult to measure under the limited privacy encountered in the field. Arm and calf circumferences correlated significantly with most of the measurements taken. In adult individuals, the calf and upper arm girths are indicators of muscle tissues and subcutaneous fat; these indices would reflect, in part, calorie and protein adequacy. Skinfold thickness is an index of leanness-fatness of individuals. Body weight, arm and calf circumferences, and skinfold thickness, however, were not significantly correlated with the mean

food energy intakes of the rural elderly. Protein and most of the nutrients examined did not correlate significantly with the anthropometric and health indices obtained in this study.

DISCUSSION

Little published information is available on the nutritional status of older people in the Philippines. In the nationwide nutrition surveys from 1958 to 1969 by the Food and Nutrition Research Center in the Philippines, limited data have been reported for the 60-and-over group (17). The clinical phase included information on men and women in this particular age group. Biochemical data were reported for the 21-and-over age group, and food consumption data were given as intake per capita.

The low body weights for heights of many older people in the sample reflects, in part, subnormal amounts of subcutaneous fat (based on low skinfold thickness) and muscle tissues (based on arm and calf circumferences). In many parts of the world, the impoverished tend to be leaner and lighter than their more affluent peers. Chronic calorie deficit would result in a constant reduction of fat and lean tissues but adjustment in physical activity prevents such losses. Although the mean food energy intakes met the allowance for calories, standard deviations were large. Energy intakes based on 24-hour recall records, however, tend to overestimate actual intake when consumption is low and to underestimate it when consumption is high (18). Some difficulties were encountered also in judging quantities of individual food items in small serving portions of mixed native dishes. Country-style meal service is the common pattern used in the rural areas. Serving dishes of food are placed on the table, and each adult person takes portions from the dish.

Data based on a single one-day recall also suggest inadequacies in good food sources of vitamin A and ascorbic acid. The food records were taken toward the end of the rainy season, and fresh fruits and vegetables generally are scarce and expensive at this time of the year. The limited distribution of vitamin A and ascorbic acid in foods is often reflected in less than adequate amounts of these nutrients in many diets. Regional surveys in the Philippines indicated low consumption of dark green and deep yellow fruits and vegetables (17).

A 24-hour food recall is considered a valid technique to observe trends in dietary patterns of groups of 50 or more persons (19), although the technique's effectiveness could be questioned when used for elderly persons with memory limitations. The ability of older people to accurately recall foods eaten can be improved, however, by careful probing during the interview (20). In the Philippines, regional dietary patterns often are based on limited amounts and kinds of foods (17), and family food intakes in a given area do not vary significantly from day to day within a given week (21). In our study, the local interviewer was properly trained and had experience in eliciting dietary information; she also was familiar with the native regional meal preparation practices and eating patterns.

To relate dietary intakes to indices of nutritional status such as anthropometric measurements, 24-hour recalls repeated at specified intervals would improve individual estimates of dietary intakes. This suggested approach may help to identify habitual patterns of intakes by elderly individuals of foods that are high in vitamin A and ascorbic acid. It also would provide information on seasonal and day-to-day variability in individual food intakes. The repeated 24-hour recalls may be validated by concurrent weighing of foods and beverages consumed by individuals in a subsample. Inclusion of appropriate biochemical indices also is essential in the assessment of nutritional status

TABLE 5

Selected Anthropometric Measurement of the Rural Elderly

Variables	Mean values ± S. D.[a]					
	Age groups, years			Age groups, years		
	60 - 69	70 and over	Total	60 - 69	70 and over	Total
n	37	18	55	30	15	45
Body weight, kg	40 ± 6	36 ± 8	38 ± 7	49 ± 9	49 ± 6	49 ± 8
Standing height, cm	147.1 ± 5.0	143.1 ± 5.6	145.8 ± 5.5	159.3 ± 6.0	159.3 ± 4.4	159.3 + 5.4
Sitting height, cm	120.5 ± 4.3	120.2 ± 4.5 (n=17)[b]	120.4 ± 4.4 (n=54)	129.0 ± 4.6	128.4 ± 4.2	128.8 ± 4.4
Upper arm, R, cm	23.6 ± 2.8	22.2 ± 3.6	23.1 ± 3.1	25.6 ± 3.6	24.6 ± 2.2	25.2 ± 3.2
Upper arm, L, cm	23.4 ± 2.8	22.1 ± 3.6	23.0 ± 3.1	25.2 ± 3.2	24.0 ± 2.7	24.8 ± 3.1
Calf, R, cm	29.3 ± 2.3	27.5 ± 2.9	28.7 ± 2.6	32.3 ± 3.3	31.4 ± 2.8	32.0 ± 3.1
Calf, L, cm	29.0 ± 2.2	27.0 ± 2.9	28.3 ± 2.6	32.3 ± 3.3	31.5 ± 3.0	32.0 ± 3.2
Skinfold thickness, mm						
Triceps, R	12.0 ± 4.0	9.5 ± 4.6	11.2 ± 4.3	7.5 ± 3.8	7.9 ± 3.6	7.7 ± 3.7
Triceps, L	12.3 ± 3.8	9.7 ± 4.1	11.5 ± 4.1	7.6 ± 3.7	7.8 ± 3.7	7.7 ± 3.7
Subscapula, R	12.7 ± 5.9	9.9 ± 6.1 (n=17)	11.8 ± 6.0 (n=54)	10.6 ± 4.3	10.3 ± 2.9	10.5 ± 3.9
Supra-iliac, R	14.2 ± 7.4	11.8 ± 7.7 (n=17)	13.4 ± 7.5 (n=54)	6.8 ± 2.1 (n=27)	9.0 ± 3.4 (n=14)	7.6 ± 2.8 (n=41)
Hand grip, kg						
R	21 ± 4	18 ± 6	20 ± 5	32 ± 8 (n=29)	29 ± 7 (n=14)	31 ± 7 (n=43)
L	20 ± 4	16 ± 5	19 ± 5	32 ± 7	25 ± 7	30 ± 8

[a] Unadjusted mean value and standard deviation.

[b] Figures in parentheses represent number of individuals observed for some measurements taken.

of the elderly. Factors that influence dietary patterns of the rural elderly in the Philippines need to be identified.

In the present study, age had a significant effect on body weight of the elderly women, but not of men. Women 70 years and older had a lower mean body weight than those less than 70. On the basis of data on about 5,500 adult Filipinos, body weights show little change between ages 25 and 65 (9). In the U.S. survey of the noninstitutionalized civilian population, adult body weights tend to increase with age up to about the sixth decade, then decline thereafter (22).

More than half the elderly participants had combined health and activity limitation scores between 3.7 and 5.0, and about one-third had ratings in the 2.4-3.6 range. The three scoring intervals used were 1.0-2.3 as Poor, 2.4-3.6 as Fair, and 3.7-5.0 as Good. The physical assessment tool was developed and used by the public health nurse in this study. Its reliability and validity as a basis for the health index and for field use by trained health workers other than clinicians need to be established. The limitation of activity status due to chronic conditions was explored as a possible health indicator of older people. There are, however, problems inherent in this interview measure of morbidity. As age increases, some reduction of activity generally is accepted. In addition, it is difficult to define activity roles that are appropriate for older people in different societies. Like the health score, the validity of the activity limitation as a health index for the elderly needs further study.

As do most of their counterparts throughout the world (14), many of the rural elderly aspire for long and comfortable lives. But concepts regarding "comfortable living" undoubtedly will vary for different societies. "Good health" did not rate high among the aspirations of the rural elderly. There is much to be learned about the lifestyle of the elderly in developing countries and its influence on their nutritional and health status. Basic knowledge is needed that is relevant to their cultural heritage. It is equally important to obtain information that will contribute to the understanding of the universal aspects of aging. An international commitment to the elderly is vital to the attainment of "health for all by the year 2000," which represents the goal of the World Health Organization (23).

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SERUM THYROXINE-BINDING PREALBUMIN: AN UNRELIABLE INDEX OF NUTRITIONAL STATUS IN CHRONIC INTESTINAL DISEASE

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ABSTRACT

Serum thyroxine-binding prealbumin (TBPA) was measured in 37 men with chronic intestinal disease and its clinical value as an index of nutritional status was determined by comparison with standard anthropometric data and serum albumin concentration. Although body weight for height, triceps skinfold, mid-arm muscle circumference and serum albumin indicated that 20% of these patients were severely and 40% were moderately malnourished, serum TBA was reduced (< 150 mg/L) in only 14%. In addition, serum TBPA did not reveal a sub-group of patients that would otherwise have remained undetected by using the other nutritional indices alone. Nevertheless, serum TBPA was significantly correlated with triceps skinfold ($P < 0.02$), serum albumin ($P < 0.05$) and overall nutritional status ($P < 0.01$) assessed by a combined nutritional score. This study indicates that serum TBPA alone is not a reliable index of nutritional status in patients with longstanding intestinal disease. The rapid and marked fluctuations of serum TBPA known to occur during fasting and re-feeding may indeed mask sub-optimal nutritional status in such patients.

KEY WORDS: Thyroxine-binding prealbumin, protein-calorie malnutrition, Crohn's disease, celiac disease.

INTRODUCTION

The nutritional consequences of chronic intestinal disease are now recognized to be an increasingly important morbidity factor (1), such that retardation of growth and development, secondary to undernutrition, may be the major presenting complaint in children with Crohn's disease (2,3) and celiac sprue (4). Physicians are gradually accepting the fact that nutritional assessment is a crucial part of the overall clinical appraisal of patients with any chronic disease, but simple sensitive tests of general nturitional status are not widely used in clinical practice.

Serum thyroxine-binding prealbumin (TBPA) concentration has been shown to be a sensitive indicator of nutritional status (5,6), an observation which has been used clinically to differentiate individuals with mild protein-energy mal-

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nutrition from normal subjects (7). Serum TBPA concentration falls rapidly during energy and protein restriction (8) and is promptly restored on refeeding (5,6,8). This sensitivity of TBPA to changes in nutritional status is largely related to its short biological half-life of 2 days and rapid turnover (9).

The purpose of the present study was to determine whether serum TBPA concentration is a useful indicator of impaired nutritional status in patients with chronic intestinal disease, such that it would detect abnormalities not apparent from standard anthropometric measurements and serum albumin concentration. Establishment of a reliable, simple and sensitive test for determining sub-optimal nutritional status in chronic intestinal disease might encourage earlier nutritional therapeutic intervention and at the same time, provide a rapid method for assessing the adequacy of appropriate replacement therapy.

PATIENTS AND METHODS

Thirty seven men with chronic intestinal disease, 15 with Crohn's disease (median age 31 years, range 15-53), all of whom had both small intestinal and colonic disease and 22 with celiac sprue (median age 42 years, range 18-71) were studied. Disease activity in men with Crohn's disease was assessed by an activity score wich took into account clinical data and some laboratory investigations (10). Scores ranged between 2-14 units and for presentation of results, patients were divided arbitrarily into those with low disease activity, 0-5 units (6 patients) and those with high activity, greater than 5 units (9 patients). Patients with celiac sprue were classified into 3 groups according to jejunal morphology, namely subtotal villous atrophy (8 patients), partial villous atrophy (8 patients) and normal mucosa (6 patients). All patients gave their informed consent before being included in the study.

NUTRITIONAL ASSESSMENT

Body weight, height, triceps skinfold (TSF) measured with Harpenden skinfold calipers, and non-dominant mid-arm circumference (MAC) were determined by standard methods (11). Mid-arm msucle circumference (MAMC) was derived from the relationship MAMC = MAC - (0.314 X TSF). Body weight was expressed as a percentage of the ideal body weight for height (11,12) and TSF and MAMC were expressed as percentages of standards obtained from the Ten-State Nutrition Survey (13). Serum albumin concentration was measured by a Vickers M300 autoanalyzer, and serum TBPA concentration by "rocket" immunoelectrophoresis (14) using rabbit anti-human prealbumin (Boehringwerke ORCA). The normal range for serum TBPA (150 - 360 mg/L, mean ± 2 SD) was established from values in 28 healthy, age-matched male control subjects. Complete nutritional data were available in 25 of the 37 patients in whom serum TBPA was determined.

An estimate of overall nutritional status was obtained by combining anthropometric measures and serum albumin concentration in individual patients using a nutritional score outlined in Table 1.

Socres for TSF and MAMC were given according to percentiles, rather than any other arbitrary measure of deviation from the median (13,15). Values at the 15th percentile were regarded as indicative of a moderate deficit and those at the 5th as representing a severe deficit. The maximum score possible from this system is 8. A score of 5 or more was regarded as a sign of severe malnutrition (i.e. at least two anthropometric measures at the 5th percentile, plus one at the 15th or an abnormality of serum albumin) and 2-4 indicating moderate malnutrition.

Differences between groups of patients were examined by the Mann Whitney U test. Correlation coefficients were determined by linear regression analysis.

TABLE 1

Nutritional Score

Factor	% Standard (percentile)	Score
Body weight/height	> 90%	0
	80 - 90% (- *)	1
	< 80% (- *)	2
Triceps skinfold	> 60%	0
	40 - 60% (15th)	1
	< 40% (5th)	2
Mid-arm muscle circumference	> 90%	0
	80 - 90% (15th)	1
	< 80% (5th)	2
Serum albumin (g/dl)		
> 3.5	100%	0
3.2-3.5	90 - 99%	1
< 3.2	< 90%	2

* Weight for height standards were not expressed in percentiles, but as percentages of ideal weights (11,12).

RESULTS

General nutritional status

Anthropometric measures ranked as percentages of standards in men with celiac sprue and Crohn's disease are shown in Table 2. Thirty percent of men had reduction in body weight of 10% or more compared to standard. A similar proportion (31%) had TSF at the 15th percentile or below. A larger proportion (77%) had MAMC at or below the 15th percentile, and 31% were at or below the 5th.

Five of the 37 patients (14%) had serum TBPA concentrations below 150 mg/L (normal range 150 - 360 mg/L) two of whom also had reduced serum albumin concentrations of 3.2 g/dl (normal range 3.5 - 5.5 g/dl). Three other patients had serum albumin concentrations of 3.5 g/dl, but serum TBPA was normal in each case (284, 228 and 172 mg/L).

Nutritional scores ranged from zero to 5, with a median score of 2. Five of the 25 patients (20%) with complete nutritional data, had nutritional scores of 5 indicating severe malnutrition, 10 (40%) had moderate malnutrition with scores of 2-4, and the remaining 40% were judged on this scoring system to be borderline or normal.

TABLE 2

Serum TBPA and Ranked Anthropometric Measurements

	Number (%) patients below standard		
	>90% Std.	80 - 90% Std.	<80% Std.
Body weight/height (n=33)	23(70)	8(24)	2(6)
TBPA <150 mg/L	1(3)	2(6)	0
	>60% Std.	40 - 60% Std*	<40% Std†
Triceps skinfold (n=26)	18(69)	6(23)	2(8)
TBPA <150 mg/L	1(4)	2(8)	1(4)
	>90% Std.	80 - 90% Std*	<80% Std†
Mid-arm muscle circumference (n=26)	6(23)	12(46)	8(31)
TBPA <150 mg/L	0	1(4)	3(12)

* Equivalent to 15% percentile; † equivalent to 5th percentile; Std., standard

Relation between serum TBPA and other nutritional indices

Prevalence of sub-normal serum TBPA concentration is included in Table 2 and is compared to the anthropometric measures ranked as percentages of standards. Serum TBPA in all instances was less commonly abnormal than deficits of TSF and AMC at each point on the ranking scale.

Correlations between serum TBPA and other measures of nutritional status are shown in Table 3. Significant positive correlation was found between serum TBPA and TSF ($P < 0.02$), and serum albumin concentration ($P < 0.05$), but not with body weight or MAMC. In addition, serum TBPA correlated significantly and negatively with nutritional score ($P < 0.01$).

TABLE 3

Correlation between serum TBPA and other nutritional indices

	Correlation coefficient r	Significance P
Body weight/height (n=33)	0.38	NS
Triceps skinfold (n=26)	0.48	<0.02
Arm muscle circumference (n=26)	0.31	NS
Serum albumin (n=36)	0.41	<0.05
Nutritional score (n=25)	-0.58	<0.01

NS - not significant

However, serum TBPA concentration did not reveal a sub-group of at-risk patients that would otherwise have remained undetected by anthropometric measurements. On the contrary, only one of 10 men with moderate malnutrition and 2 of 5 with severe malnutrition (according to the nutrition score) had serum TBPA concentrations below 150 mg/L. However if albumin concentrations of $\leq$ 3.5 g/dl were taken into account, 4 of the 5 severely malnourished males would have been detected by the combination of these two variables.

Serum TBPA and disease activity

Serum TBPA was significantly lower in untreated celiacs with subtotal villus atrophy (median 170 mg/L, range 119 - 246) than celiacs with partial villus atrophy (median 257 mg/L, range 172 - 294; $P < 0.02$) and those with normal villi (median 217 mg/L, range 170 - 378; $P < 0.02$). Similarly, patients with high activity Crohn's disease (activity score < 5 units) tended to have lower serum TBPA concentrations (median 182 mg/L, range 34 - 365) than those with low disease activity scores (median 230 mg/L, range 170 - 284) although this failed to achieve statistical significance. Serum TBPA was, however, significantly correlated with Crohn's disease activity score ($r = -0.54$; $P < 0.05$).

DISCUSSION

Previous studies have clearly shown that serum TBPA concentration changes rapidly with alterations in the protein and particularly, the energy content of the diet (6,8). Observations in children with PCM (5,7) and adult surgical patients (16) suggest that serum TBPA is a more sensitive indicator of impaired nutritional status than serum albumin and anthropometric measures. However, in the present study of patients with two chronic intestinal diseases, Crohn's disease and celiac sprue, serum TBPA alone was surprisingly unhelpful in revealing at-risk patients with borderline or moderate malnutrition. Only 10% of patients with moderate malnutrition had serum TBPA concentrations below 150 mg/L, the lower limit of the normal range. The normal range of serum TBPA in the present study is similar to that reported previously (6,7,16). Fortuitously, this unselected group of patients included a large proportion of subjects with borderline or moderate malnutrition, thus increasing the demands on the sensitivity of indices of nutritional status, whereas previous studies included a greater proportion of severely malnourished subjects (6,7).

One possible explanation for the divergance between the finding of this study and those reported previously, is that the majority of these apparently sub-optimally nourished patients with normal serum TBPA were in fact, not malnourished. This would seem unlikely, since the assessment criteria in the present study are those that are now generally accepted (11,12,13,15,17). However, the importance of the choice of nutritional classification has been dramatically shown in children with PCM (7), such that normal and mildly malnourished subjects by the Waterlow classification (18) could be clearly distinguished by serum TBPA, whereas overlap between equivalent categories occurred if the Wellcome classification (19) was used.

Nevertheless in the present study, serum TBPA concentration was related to other nutritional parameters, indicated by significant correlations with nutritional score and TSF, although not with MAMC or body weight alone, as reported previously (7,16). In agreement with a study in surgical patients (16) serum TBPA was also significantly correlated with serum albumin concentration. In addition, serum TBPA was clearly related to jejunal morphology in celiac sprue and to the Crohn's disease activity score in patients with Crohn's disease.

The reason why serum TBPA should, against persuasive contradictory evidence in the literature, be unhelpful in detecting nutritionally at-risk patients with chronic intestinal disease is intriguing. One important factor may be the duration and severity of protein and energy restriction. Although objective dietary assessment was not made in these patients with intestinal disease, the majority were attending the out-patient department in a stable clinical state, and there were no indications of abrupt changes in dietary intake. However, all three of the patients with Crohn's disease with reduced serum TBPA had been admitted acutely to hospital in relapse, two of whom had sub-acute intestinal obstruction. One might speculate that reduced serum TBPA in these patients was related to acute protein or more likely energy restriction, since serum TBPA appears most sensitive to reduction in energy intake (8). This argument can be extended further to suggest that mild, chronic undernutrition may not necessarily result in reduction of serum TBPA, although as in these patients with intestinal disease, it may be sufficient over a long period to reduce fat stores and decrease somatic protein. This postulate is supported by the observation that serum TBPA concentration returns to normal during the second and third week of nutritional rehabilitation of children with severe PCM (6), a time when serum albumin and anthropometric measures are still abnormal. Similarly, following a period of acute protein and energy restriction, serum TBPA concentration in obese women is restored after only three to four days of nutritional rehabilitation (8).

Paradoxically, serum TBPA alone may be too sensitive to be useful clinically in the assessment of nutritional status in chronic intestinal disease, since the rapid changes in serum TBPA that can occur with fluctuations in dietary protein and energy intake during relapse and remission of inflammatory bowel disease will not necessarily reflect general background nutritional status, in the same way that serum carotenoids may parallel recent dietary intake, rather than body stores. A relatively brief period of refeeding may increase serum TBPA to normal, misleadingly indicating that nutritional rehabilitation is complete (6).

Undoubtedly, when serum TBPA is decreased the physician should be alerted, but not complacent when levels are normal in patients with chronic intestinal disease. Serum TBPA would seem to be of value in monitoring nutritional therapy in the short-term, but for the reasons already stated should not be viewed in isolation. In the present study, combining the results of a rapid turnover protein, TBPA, with serum albumin (half life $\sim$ 20 days) (20) increased the likelihood of detecting moderate malnutrition in patients with chronic intestinal disease. Similarly, pre-operative nutritional assessment of surgical patients was most complete by combining TBPA with anthropometric and other data (16).

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EFFECT OF VARIABLE AMOUNTS OF PROTEIN INTAKE ON URINARY CALCIUM EXCRETION IN YOUNG NIGERIANS

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ABSTRACT

The effect of various levels of low protein intake on urinary calcium excretion was studied in eight young adults. The study lasted 54 days and consisted of four experimental periods, each of 10 days, during which subjects were fed four different levels of protein (0.30, 0.45, 0.60, and 0.75g/kg body weight/day) and adequate energy (45kcal/kg body weight). Average daily calcium and phosphorus intake at the four different levels of protein intake were 550, 860; 600, 866; 625, 916; 615, 930 mg respectively. Daily urine samples which were collected during the last five days of each experimental period were analysed for calcium content. Urinary calcium levels were relatively low. However, regression analysis showed a significant relationship between the level of protein intake and urinary calcium excretion. Analysis of variance also showed significant group and individual differences in urinary calcium excretion in response to changes in the level of protein intake.

KEY WORDS: Dietary protein, protein deficiency, urinary calcium.

INTRODUCTION

Several studies have been carried out on the influence of varying protein intake on calcium retention, absorption and excretion. Several workers (1-7) have shown that an increase in protein intake is usually accompanied by an increase in urinary calcium level. In the studies by Walker and Linkswiler (5) young men consuming diets of different protein level were observed to excrete increasing amounts of calcium in their urine as the intake of protein was raised. At a protein level of 142g/day, the calciuretic effect was accompanied by a negative calcium balance. Other studies by Margen et al (7) also showed a positive correlation between protein intake (ranging from 0 to 96gN/day) and calcium excretion.

In all these studies, the maximum levels of protein tested in each case was higher than habitual intake levels. Protein intake in Nigeria as in most developing countries of the world (8) is relatively low. It is not known if calcium excretion as induced by different levels of protein intake can be markedly increased if intake levels were limited to such low levels as obtained among Nigerians of the low socio-economic group.

This paper reports the effect of different levels of low protein intake on the urinary calcium excretion of eight young adult men. The men were fed at different periods, protein levels of 0.30, 0.45, 0.60 and 0.75g/kg body weight/day. The habitual protein intake of these subjects ranged between 0.78 and 0.96g/kg body weight/day.

MATERIALS AND METHODS

Subjects. The eight subjects participating in this study were all young male Nigerians in their third year in a Medical School. They were between 20 and 23 years old and had initial body weights ranging from 54.48 to 64.14 kg. They were given a comprehensive medical examination and were found to be fit. Subjects lived in nearby dormitories and were engaged in their normal daily routine activities during the period of the study.

Design of experiment. The subjects were fully briefed on the purpose, nature and design of the study. The study lasted 54 days and consisted of four experimental periods each of 10 days during which subjects were fed different levels of protein (0.30, 0.45, 0.60 and 0.75g/kg) in their diets. A protein-free diet was given for one day before the beginning of each period so as to promote rapid adaptation to experimental diets. The order of feeding the four levels of protein to each of the subjects is as given in Table 1. The order of feeding was such that 50% of the subjects were assigned to a descending sequence of protein intake i.e. 0.75, 0.60, 0.45 and 0.30 g protein/kg body weight while the rest were on ascending sequence. Thus, within each experimental time period, there were two relatively different levels of protein intake - a 'high' and 'low' level. In each of the four experimental periods, the first five days were allowed for adaptation and the remaining 5 days for the collection of urine. Between each study period, there was a 3-day break during which subjects were free to eat any food of their choice.

TABLE 1

Sequence of protein intake (g protein/kg body weight/day) of each subject during the study

Subject Code No.	Experimental Period			
	1	2	3	4
1SO	0.30	0.45	0.60	0.75
2AS	0.30	0.45	0.60	0.75
3FA	0.75	0.60	0.45	0.30
4KU	0.30	0.45	0.60	0.75
5OM	0.75	0.60	0.45	0.30
6OP	0.30	0.45	0.60	0.75
7DU	0.75	0.60	0.45	0.30
8HA	0.75	0.60	0.45	0.30

Diets. Diets fed during each of the experimental periods were based on staple foods usually consumed by the subjects. These were bread, margarine, custard, vegetable, cassava meal, boiled yam, beef, egg and oranges. Protein intake level during the first experimental period was contributed to mostly by the consumption of beef. During the subsequent periods a uniform amount of egg was included in the diets while the amount of beef was varied to make up the desired protein intake level. Energy intake was maintained in each period at 45Kcal/kg body weight. In order to obtain this desired energy level without exceeding protein intake levels, "Caloreen" which is a glucose polymer, was added in appropriate amounts to the meals which were served. The average calcium intake during each of the four levels of protein intake were low and very similar (550, 600, 625, 615mg). Phosphorus intake levels at the different protein intake levels were also low and did not show any wide variation (860, 866, 916, 920mg). Subjects were made to understand that they were limited only to the experimental diets provided each day.

Urine collection. During the last five days of each experimental period, 24-hour urine samples were collected in plastic urine bottles containing 5ml 1 NHCl. The volume of each 24-hour urine collection was measured and an aliquot was taken and stored at -20°C until required for analysis. As a check for total urine collection, a 24-hour urinary creatinine determination was carried out on individual samples.

Analytic methods. The five urine samples collected during each 24-hour period were subsequently pooled in proportion to their original volumes and analysed for their calcium content using the Technicon SMA auto-analyser method. The mean values and the standard deviations were calculated and results were subjected to the paired Student's t-test; an analysis of variance as well as a simple regression analysis. Values with $p < 0.05$ were regarded as significant.

RESULTS

Throughout the duration of the study, anthropometric measurements of the subjects did not show any significant changes. Table 2 shows the mean urinary calcium excretion of individuals in the ascending (A) and the descending (D) sequence of protein intake at the different periods of the experiment. Significant differences ($p < 0.05$) occurred only between the 0.30 and 0.75g protein/kg levels. Regression analysis of average urinary calcium excretion against mean protein intake produced a linear curve with the equation: $Y=10.0+29.5X$ (Fig. 1).

The relationship was statistically significant ($r=0.98; p < 0.01$). As shown in Table 3, all subjects (except subject No. 60P) excreted more calcium in their urine when the protein intake level was higher than the minimum level of 0.30g/kg/day. However, the mean calcium excretion at 0.45g protein/kg/day intake was not significantly different from that at the minimum protein intake level, but there was a significant difference at 0.60g/kg/day intake ($p < 0.01$) and at 0.75g/kg/day ($p < 0.001$). Expressing individual calcium excretions as percentages of that excreted during the lowest level of protein intake at 0.30g/kg/day, it was observed that individual urinary calcium increased by 26 to 183 percent (average 80 percent) when protein intake was increased from 0.30 to 0.75g protein/kg/day. Thus there was a wide individual variation in the response to increased protein intake.

TABLE 2

Mean 24-hour urinary calcium excretion (mg) during each experimental period

Group	Subject	Experimental Period 1	2	3	4
Descending sequence	3FA	43.6	37.0	20.6	15.4
	5OM	22.2	25.9	20.9	17.0
	7DU	24.7	23.8	27.3	13.0
	8HA	31.7	32.9	37.8	25.2
	Mean	30.6±9.6	29.9±6.1	26.7±8.1	17.7±5.3
Ascending sequence	1SO	17.1	19.7	27.1	35.8
	2AS	20.3	30.2	32.1	31.6
	4KU	16.3	21.2	19.2	26.2
	6OP	17.8	17.6	31.8	32.9
	Mean	17.9±1.7	22.2±5.6	27.6±6.0	31.6±4.0

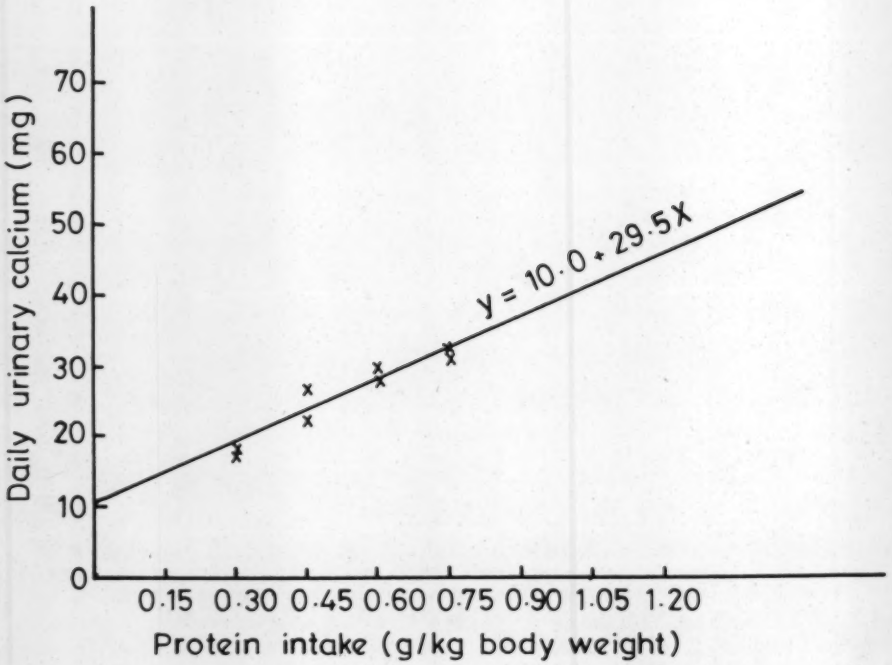

Fig. 1 Regression of average urinary calcium excretion against mean protein intake.

TABLE 3

Effect of protein intake level on urinary calcium concentration (mg/24-hour)

Subject	Protein intake level (g/kg body weight/day)			
	0.30	0.45	0.60	0.75
1SO	17.1	19.7 (15%)	27.1 (58%)	35.8 (109%)
2AS	20.3	30.2 (49%)	32.1 (58%)	31.6 (56%)
3FA	15.4	20.6 (34%)	37.1 (140%)	43.6 (183%)
4KU	16.3	21.2 (30%)	19.2 (18%)	26.2 (61%)
5OM	17.0	20.9 (23%)	25.9 (52%)	22.2 (31%)
6OP	17.8	17.6 (-1%)	31.8 (79%)	32.9 (85%)
7DU	13.0	27.2 (109%)	23.8 (83%)	24.7 (90%)
8HA	25.2	37.8 (50%)	32.9 (31%)	31.7 (26%)
Group	17.8*	24.4	28.7	31.1*
Mean±SD	±3.7	±6.8	±5.8	±6.8

Analysis of Variance

Source of variance	DF	SS	MS	F
Dietary protein level	3	821.6	273.9	11.01*
Subject	7	456.0	65.1	2.62@
Error	21	522.2	24.9	1.0
TOTAL	31	1799.8		

Figures with the same sign in a row are significantly different from each other. *=$P < 0.001$; @=$P < 0.01$. Figures in parenthesis show percentage increase in urinary calcium above that at the lowest level of protein intake.

DISCUSSION

The result obtained in this study suggest that there is a positive relationship between the different low levels of protein consumed and urinary calcium levels. The results thus agree with those of previous studies reported in the literature which show that increased protein intake lends to an increase in urinary calcium levels (2-8, 10-14, 17, 18, 20). However, unlike most of the studies reported in the literature the magnitude of increase in urinary calcium excretion induced by increases in the level of protein consumed was relatively smaller. The absolute amounts of urinary calcium were also low and differed significantly between individuals and the different levels of protein consumed. The observation of such individual differences in urinary calcium excretion in response to protein intake, has been reported in the literature. For example, in one of the several studies carried out by Margen et al (7) adults fed less than 1gN/day and 1.6g calcium were observed to excrete an average of 46mg calcium in their urine with individual values ranging from as low as 17mg to 83mg/day. When the level of protein was increased to 12-13gN/day (over 1000% increase), mean urinary calcium excretion increased to 92mg (an increase of only 100%) with individual levels ranging from as low as 25mg to 172 mg/day.

Our present results thus suggest that subnormal levels of protein intake is accompanied by relatively low levels of urinary calcium. Using the lowest level of protein intake as the basal point of reference, an average of 80 percent increase was observed in urinary calcium level when the protein intake was increased to 0.75g/kg. On the other hand regression analysis (involving all the different levels of protein tested and the respective urinary calcium levels) show that for a 150 percent increase in protein intake, urinary calcium increased by 70 percent. Hegsted et al (20) using data from a number of different studies in which urinary calcium excretion in response to different levels of protein was measured, demonstrated that for a 100 percent increase in protein intake urinary calcium increased by 50 percent. Based on the regression equation they obtained from this relationship, a 150 percent increase in protein intake in the present study should result in an increase of 75 percent in urinary calcium excretion. Our regression equation gives an increase of 70 percent. Thus urinary calcium excretion in response to changes in protein intake was relatively lower. This difference in response is expected since according to Hegsted et al (20) the linear relationship they established through a regression analysis between protein intake and urinary calcium excretion is valid only within a range of adequate to very high protein intakes. Our range of protein intakes were all below recommended dietary allowance and therefore inadequate. The results thus suggest that urinary calcium excretion in response to different levels of inadequate protein intake is relatively lower than that commonly observed in different levels of adequate to high protein intake.

The increase in urinary calcium as induced by an increase in protein intake has been associated with changes in kidney function. According to earlier studies (9) an increase in protein intake causes an increase in glomerular filtration in man. Other metabolic studies carried out in humans have also indicated that an increase in dietary protein leads to an increase in glomerular filtration rate and calcium clearance as well as a reduction in the fractional tubular reabsorption of filtered calcium (10, 11, 12). The occurence of calciuria in high protein intake has therefore been explained as being due to these changes in kidney function. In particular, it is now believed that the cause of the calciuria following the ingestion of high protein is due mostly to decreased tubular reabsorption of calcium (10-13). According to Schuette et al (11) the reduction in renal tubular reabsorption may be due to an increase in the hydrogen ion load on the renal tubular cells. In fact earlier reports in the literature (14) had shown that chronic metabolic acidosis directly inhibits

renal calcium reabsorption. Since it has been shown that high protein diets produce an increase in renal acid excretion (15, 16) it follows that very low or inadequate levels of dietary protein such as those consumed in the present study will result in relatively very low renal acid load and hence produce a relatively minimal effect on renal tubular reabsorption of calcium.

Another factor that has been implicated in the hypercalciuria of high protein diets is the production and excretion of sulphate. The catabolism of sulphur amino acids is a major source of endogenous acid (16) and a significant correlation has been observed between the increase in urinary calcium excretion and the increase in sulphate excretion following increased level of protein intake (11, 17). Thus, as shown by Whiting et al (17) the variability of the calciuretic effect of different proteins can be significantly influenced by the variation in the sulphur amino acid content of the proteins consumed. Most Nigerian diets are plant based and it is likely that they have disproportionate amounts of these amino acids which affects the total amount of sulphated and renal acid and hence the amounts of calcium excreted in urine.

Besides the influence of increased protein intake, changes in calcium intake may also increase urinary calcium content (18). However, as noted by Margen et al (7) the effect of increased calcium intake on the urinary calcium excretion cannot be easily explained if increased intake level is below requirement levels. In the present study, average dietary calcium levels was low (596mg/day) and ranged between 550 and 620mg.

Other dietary factors have also been implicated as having an effect on calcium metabolism and hence on urinary calcium excretion. Some studies reported in the literature suggest that an increase in dietary phosphorus levels decreases urinary calcium excretion (19, 20, 21). However, the simultaneous increase in protein and phosphorus intake has a much less dramatic effect on urinary calcium than an increase in protein intake alone (20). The levels of phosphorus in the diets consumed by subjects in the present study were relatively low and constant and so probably did not exert much influence on urinary calcium levels. The level of protein or fiber present in a diet can also affect the intestinal absorption of calcium. Reports in the literature indicate that high protein intake improves calcium absorption (3, 4, 5) while high dietary fibre (22, 23) has an opposite effect. Since the diets consumed in this study were plant based, it is likely that their relatively high fiber content and the low levels of protein affected calcium absorption. The relative contribution of such effects on the observed urinary calcium levels was, however, not measured.

Recent reports by Allen et al (24) suggests that insulin secretion may be involved in the protein induced mechanism that leads to increased calcium excretion. The authors reported that there was a significant correlation between post prandial increases in insulin secretion and urinary calcium excretion when protein intake of adults was increased three fold. Earlier reports in the literature show that malnutrition or the level of dietary protein affects insulin secretion in man (25, 26). In particular, protein deficiency in the rat (27) and in the pig (28) has been reported to lead to a decrease in insulin secretion. Based on these reports, it is likely that the low levels of protein consumed in the present study induced relatively low insulin secretion and hence its limited effect on urinary calcium excretion.

In conclusion, results of the present study suggest that intakes of different levels of low protein such as do occur in the diets of Nigerians from the low socio-economic group is accompanied by significant differences in urinary calcium excretion. The absolute amounts of urinary calcium were however low. Since reports of human studies indicate that the calciuretic effect of

high protein intake can lead to a negative calcium balance (11, 12) and animal studies further reveal that high protein and low calcium diets result in a high inhibition of bone formation (29) the present results may be of wide implication in Nigeria particularly in the dietary management of hospitalised patients with fractures from the low socio-economic group. Diets usually given to such patients in the hospitals are nutritionally better (with a relatively high protein content) than habitual diets consumed by the patients in their homes before hospitalisation. We speculate that such differences in dietary protein levels might have a delaying effect on healing of fractures. We are currently carrying out a study to investigate this.

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BIOTIN CONTENT OF HUMAN MILK DURING EARLY LACTATIONAL STAGES[1]

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ABSTRACT

In order to expand and update the nutritional information of human milk, the biotin content was determined in early transitional (three to eight days postpartum), transitional (10 to 14 days postpartum), and mature (30 to 47 days postpartum) human milk from 84 donors. The women ranged in age from 16 to 38 years. The biotin level increased significantly ($P < .01$) as time postpartum increased from 0.07 µg/100 g at the early transitional stage to 0.30 and 0.47 µg/100 g at the transitional and mature stages, respectively. Early morning and late evening samples were collected at the transitional and mature stages; however, no significant diurnal variations were observed.

Key terms: human milk, biotin, lactation.

INTRODUCTION

The infants requirement for biotin is not known; however, in 1976 the Committee on Nutrition of the American Academy of Pediatrics recommended that infant formulas contain a minimum of 1.5 µg of biotin per 100 kcal. This level is similar to the biotin content of commonly used milk-based formulas and represents an intake that is adequate to avoid any signs of biotin deficiency (1).

While breast-fed infants generally exhibit lower incidence of gastrointestinal infections than bottle-fed infants (2, 3), it has also been reported that sudden infant death syndrome (SIDS) is less common among breast-fed infants (4). A recent report that diets marginally deficient in biotin, coupled with stress, may be linked to SIDS (5), suggests that the biotin content of an infant's diet may have more importance than previously recognized. The recent concern over biotin in the infant's diet was reflected in the Infant Formula Act of 1980 (6) which established 1.5 µg/100 kcal (1.05 µg/100 g) as the minimum biotin content in non-milk based formulas. Previous standards, such as the 1971 FDA regulations for infant formulas (7) did not set minimum biotin standards.

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The major objective of this study was to obtain current data on the biotin content of human milk during early stages of lactation. Further, diurnal variation in the biotin content was assessed. This information is needed for a better understanding of the biotin requirements of infants.

MATERIALS AND METHODS

Description of Donors: The donors for this study were all residents of the Athens, Georgia area. They were contacted initially one day postpartum at either of the two major hospitals in Athens. Over a 2.5 year period, a total of 87 women agreed to participate and signed an informed consent. The women were of middle socio-economic status and ranged in age from 16 to 38 years. Of the women who participated, 79 were Caucasian, 3 were black, and 2 were of Oriental descent. All of the women were in good health, encountered no unusual difficulties during delivery, and delivered healthy full-term infants.

Collection of Milk Samples: Donors collected the milk samples by manual expression or by using a Lopuco breast pump (Lopuco Ltd., Woodbine, MD). The milk was collected at the following three stages of lactation: early transitional (three to eight days postpartum), transitional (10-14 days postpartum), and mature (30-47 days postpartum). At the early transitional stage, each donor collected one composite sample from both the late evening and early morning feedings. Two samples were collected at the transitional and mature stages. For these samples, donors were instructed to collect approximately equal volumes of milk from the beginning, middle, and end of one late evening and one early morning feeding. The samples were refrigerated immediately after collection, picked up after the morning collection, and transported on ice to the laboratory.

Biotin Assay: Biotin content was determined by a microbiological assay adapted from the procedure of Skeggs (8) using Lactobacillus plantarum ATCC 8014 as the test organism. Milk samples were diluted with an equal volume of 6N H_2SO_4 and autoclaved at 20 psi, 120°C for 60 min. After hydrolysis, the extraction medium was cooled, adjusted to pH 4.5 with 1.0 N NaOH and clarified by filtration. Final dilution of the extract was completed with distilled water. The assay was run on two or more sample aliquot volumes using Difco biotin assay medium (Difco Laboratories, Detroit, MI). After incubation at 37°C for 16-20 h, absorbance of the growth medium was determined at 620 nm in a Bausch and Lomb Spectronic 20 Spectrophotometer (Bausch and Lomb, Inc., Atlanta, GA). Growth response was proportional to the volume of extract assayed. Recovery of added biotin averaged 110%.

Statistical analysis was performed using the general linear models procedure and Duncan's multiple range test in the Statistical Analysis System (9) package.

RESULTS AND DISCUSSION

Data showing the mean biotin content of early transitional, transitional and mature milk are presented in Table 1. No significant ($P < .05$) diurnal variations were noted at either the transitional or mature stages. For all additional statistical comparisons the morning and evening results were combined. The biotin content was the lowest in early transitional milk (0.07 µg/100 g) and increased significantly ($P < 0.01$) to 0.30 µg/100 ml at the transitional stage and 0.47 µg/100 ml at the mature stage. Macy (10) reported similar biotin levels in early lactational stage milk. She reported a biotin content of 0.06 µg/100 ml in colostrum (one to five days postpartum) and 0.35 µg/100 ml in transitional milk (six to 12 days postpartum); however a higher level of 0.81 µg/100 ml was

reported for mature milk collected 15 days to 15 months postpartum. Since a study by Hood and Johnson (11) indicated that the biotin content of human milk continues to increase as lactation time increases, the long range of lactation time represented by Macy's (10) compilation of data may account for the higher biotin value at the mature stage. Hood and Johnson (11) found that the biotin content increased rapidly between day one and day seven from 0.30 to 0.68 µg/100 ml followed by an increase to 1.25 and 1.27 µg/100 ml at days 49 and 70, respectively. The fact that these values are much higher than the results reported by Macy (10) and in our study could be due to geographical location and/or differences in diet. Another study (12) on samples collected in five separate locations in Great Britain, but all transported to one laboratory for analysis, reported large geographical differences with mean values ranging from 0.52 µg/100 ml in one area to 1.13 µg/100 ml in another area. A mean of 0.72 µg/100 ml was reported for samples from each of the other three locations, resulting in an overall average of 0.76 µg/100 ml. These mean values were determined on pooled samples of mature milk collected 14-63 days postpartum.

TABLE 1

Mean Biotin Content of Early Transitional, Transitional and Mature Human Milk

Lactational Stage*	Mean ± SD** (Range) µg/100 g
Early transitional n = 84	0.07 ± 0.09 a (0.00 - 0.72)
Transitional n = 67	0.30 ± 0.22 b (0.01 - 1.18)
Mature n = 64	0.47 ± 0.22 b (0.05 - 1.41)

* n denotes the number of donors

** Means followed by different postscripts are significantly different at $P < .01$ as determined by Duncan's multiple range test.

The frequency distribution of the biotin values are shown in Fig. 1. As time postpartum increased, the range of biotin values also increased. At the early transitional stage, 95% of the samples were in the range 0.0-0.2 µg/100 g, with 3% in the range 0.2-0.4 µg/100 g and 2% in the range of 0.6-0.8 µg/100 g. The range at the transitional stage was from 0.01-1.2 µg/100 g with 95% of the samples ranging from 0.01-0.7 µg/100 g. The remaining 5% were in the range from 0.7-1.2 µg/100 g. At the mature stage, 95% of the samples ranged from 0.05-1.0 µg/100 g while the remaining 5% ranged from 1.0-1.4 µg/100 g.

Although a Recommended Dietary Allowance (RDA) for biotin has not been established, the Committee on Dietary Allowances estimated the safe and adequate intake of biotin for infants up to six months of age to be 35 µg/day (13). Based on an estimated average daily consumption of 600 ml of milk (14) for infants one month old, the infants in the present study consumed an average of 2.8 µg biotin/day. This level is considerably lower than the 6.3 µg/day provided by infant formulas containing a minimum of 1.5 µg/100 kcal and more than ten-fold lower than the estimated safe and adequate level recommended by the National Research Council (NRC) (13). Therefore, these findings indicate that there is a large difference between the estimated safe and adequate level suggested for biotin by the NRC and the actual intakes of beast-fed infants during the neonatal period.

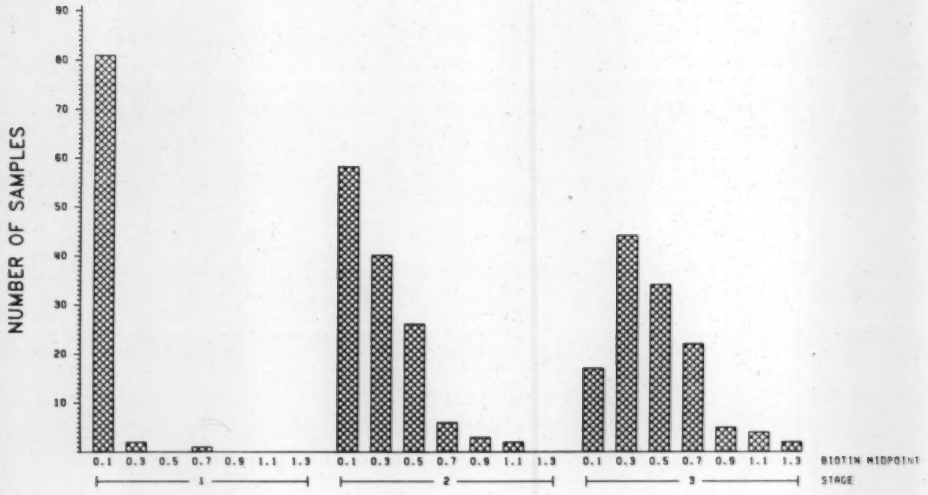

FIG. 1

Biotin frequency distribution for early transitional, transitional, and mature milk (stages 1, 2, and 3, respectively)

With respect to the report by Johnson et al. (5) that SIDS may be linked to biotin deficiency, the evidence here seems to contradict their theory. As mentioned earlier, SIDS is less prevalent among breast-fed infants (4), yet our study shows that the biotin concentration of human milk is one-half of that required in infant formula. If in fact biotin deficiency is linked to SIDS, breast-fed infants with a lower biotin intake would be more susceptible than formula-fed infants.

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ZINC HAIR CONCENTRATIONS AND ESTIMATED ZINC INTAKES OF FUNCTIONALLY DELAYED NORMAL SIZED AND SMALL-FOR-AGE CHILDREN

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ABSTRACT

The relationship of hair zinc concentrations and dietary zinc intakes to height and weight were compared among three groups of children: small-for-age and normal sized functionally delayed children, and normal sized nondelayed children. There were no statistical differences among the groups for either hair zinc concentrations or the dietary zinc intake. The heterogeneity of hair zinc concentrations in the small-for-age groups prevents making generalizations about the relationship between hair zinc and growth status in functionally delayed children.

Key Words: Zinc, Growth, Hair Zinc, Dietary Zinc Intake

INTRODUCTION

A large number of developmentally delayed children are small-for-age relative to the present standards for the general population (1-3). The wide variety of diagnostic categories of developmental delays has complicated efforts to identify the causes of poor growth in these children. Functional developmental delay, is a category of developmental delay without an identifiable specific organic cause. Functional developmental delays suggest the possibility of environmental factors, including nutrition, as having a role in the growth retardation observed in these children.

This study compared the hair zinc concentrations and dietary zinc intakes among small-for-age and normal sized functionally delayed and normal sized nondelayed children to determine if poor zinc nutriture is related to the impaired growth seen in small-for-age functionally delayed children.

METHODS

With informed consent, 42 children, ranging in age from 3.0 to 9.0 years, living in the Washington, D.C. area, were assigned to one of three equal sized groups according to level of cognitive functioning and size. Criteria for participation of the developmentally delayed subjects included: a previous diagnosis of developmental delay, confirmation of this diagnosis by an I.Q. score less than

80 on a standardized intelligence test, and an undetermined cause of the developmental delays after medical examinations. The developmentally delayed subjects were divided into two groups: 1) small-for-age: having height, weight, or weight-for-height less than the 5th percentile values on the growth charts constructed by the National Center for Health Statistics; and 2) normal sized: having height, weight and weight-for-height values between the 25th and 75th percentiles. All of the developmentally delayed subjects were free of severe motor impairments to which retarded growth could be primarily attributed, and none were living in a group care facility.

The developmentally delayed subjects were identified at the Georgetown University Child Development Center, where they were participating in a collaborative research project approved by the hospital research committee.[1]

For the nondelayed group, subjects were normal sized, as defined above, and of normal intelligence, as defined by an I.Q. score greater than 100 on a standardized intelligence test. Age range, sex and racial composition, and a socioeconomic levels of this group were matched as closely as possible to those of the two groups of developmentally delayed subjects. Nondelayed subjects participated under procedures approved by the Committee on Human Subjects at the University of Maryland.

Hair samples were clipped close to the scalp in the suboccipital region. The proximal 5 cm of the samples were retained and stored in sealed plastic envelopes prior to analysis, and then were washed and analyzed according to procedures adapted from McBean et al. (4).

For each day's batch of hair samples being analyzed, zinc concentrations were also determined in sample blanks and 50 mg samples of National Bureau of Standards Bovine Liver Standard Reference Material 1577. Accuracy of hair zinc concentration values was thus monitored by comparison to the analytical values obtained for the liver samples, which were always within the acceptable range (130 ± 13 µg/g zinc).

Mean dietary intake of kilocalories, protein, and zinc for each of the groups was estimated from 24-hour recalls obtained by a nutritionist from a parent of each subject. None of the subjects were taking supplements which contained zinc. Food models, measuring spoons and cups, measured tableware, and consistent verbal prompts were used during the interviews to enhance the accuracy of the recall information. Kilocalorie and protein intakes were calculated from U.S. Department of Agriculture nutrient composition handbooks (5, 6) and, where necessary, from manufacturers' product analysis data. Zinc intakes were derived from published tables on the zinc content of foods (7-10).

Data were analyzed by a one-way analysis of variance, to determine if statistical differences ($p < .05$) existed among the three groups of children (11). Also correlation analyses were done (11).

RESULTS

The means of age, height and weight and hair zinc concentration of each of the groups are presented in Table 1.

1. MC-R-390-400, MCH, USPHS, HHS. The Ohio State University Research Foundation.

TABLE 1
Age, Height and Weight, and Hair Zinc Concentrations of Groups

Group*	Age	Height	Weight	Hair Zinc
	mos.	cm.	kg.	μg/g
Functionally Delayed				
Small-for-age	66±6†	101.4±3.1	15.2±0.9	138±16
				(67-276)
Normal sized	72±5	113.0±2.6	20.1±1.0	119±11
				(64-187)
Nondelayed				
Normal sized	65±6	111.2±2.6	19.9±1.4	96±11
				(31-168)

*n = 14 for all groups
† Means±SEM
Range of values in parentheses

The mean zinc concentration was highest in the small-for-age group: 138±16 μg/g (mean ± SEM); lowest for the nondelayed group: 96±11 μg/g; and intermediate for the normal sized delayed group: 119±11 μg/g. The difference between the high and low means was not significant at $p < 0.05$. There was no correlation between age and hair zinc concentrations.

Dietary data are summarized in Table 2. The mean intakes of zinc, calories, and protein were highest for the normal sized retarded subjects: 9 mg, 1800 kcal, 65 g, respectively; lowest for the nondelayed subjects: 7 mg, 1600 kcal, 56 g respectively; and intermediate for the small-for-age subjects. Although the trend with respect to zinc intake was similar to that of the hair levels, with the nondelayed children having the lowest mean zinc intake as well as hair level, the difference in intake among the groups were not significant. Further, there was no significant correlation between zinc intake and hair zinc level in any of the groups. Differences in energy and protein intakes among groups were also not significant.

TABLE 2
Zinc, Energy and Protein Dietary Intake Data for Groups

Group	Dietary Intake		
	Zinc†	Energy	Protein
	mg/day	kcal/day	g/day
Functionally Delayed			
Small-for-age	8±1*	1700±190	62±7
	(3-18)	(700-2800)	(30-110)
Normal sized	9±1	1800±220	65±6
	(4-19)	(1100-4400)	(31-109)
Nondelayed			
Normal sized	7±1	1600±100	56±5
	(4-12)	(1200-2400)	(34-106)

*Mean±SEM, range in parentheses.
† The RDA is 10 mg

DISCUSSION

The heterogeneity of the hair zinc concentrations in the small-for-age group, evidenced by the broad range of values, precludes making generalizations about the relationship between hair zinc and poor growth in these functionally delayed children. Within this group, the five values less than 105 μg/g zinc may be indicative of marginal zinc status. For these children, additional tests of zinc nutriture would be warranted. At the other extreme of the range for this group were the three hair concentrations greater than 200 μg zinc/g hair. Such high values may result from slowed hair growth in conjunction with the poor physical growth. When minerals continue to be deposited in a slowly growing hair shaft, mineral accumulation results, and normal or elevated concentrations may be observed (12). Such a phenomenon has been described in the severe zinc deficiency seen in patients with untreated acrodermatitis enteropathica (13), and in cases of protein-calorie malnutrition (14). Ertenet al. (15), studying the zinc status of protein-calorie malnourished children, reported a range of hair zinc concentrations (79-277 μg/g) similar to that found in the small-for-age group in this study.

In the nondelayed group, eight of the 14 subjects had hair zinc levels in the marginal or deficient ranges. Although such low values are suggestive of suboptimal zinc nutriture, that interpretation is complicated by these subjects' apparent health and normal size. If their zinc status was deficient, the condition had not persisted long enough or been severe enough to exhibit clinical symptoms. An alternative explanation for these relatively low hair zinc concentrations would be that the hair zinc reflects metabolic needs. An inverse relationship between growth velocity and plasma zinc concentration has been reported by Butrimovitz and Purdy (16). Their data suggest that rapid periods of growth place, an additional drain on zinc reserves. In periods of active growth where the metabolic demand is high, there may be less zinc available for deposition in the hair shaft.

The estimated mean daily intake of zinc did not differ significantly among groups, and was apparently adequate in relation to the RDA, which is 10 mg per day for this age range (17). The lowest mean intake, that of the non delayed, provided approximately two thirds of the RDA. These intake levels are similar to those reported by other investigators (18, 19).

For all of the groups, analysis of the relationship of the hair zinc levels to the dietary zinc intakes showed no significant correlation, which has also been reported by other investigators (20,21). One potential factor contributing to such a lack of correlation would be in the case of a low dietary zinc intake being associated with a relatively high hair zinc concentration, where hair growth has been slowed. In contrast, a relatively low dietary zinc intake may also be associated with a low hair zinc level, as seen in chronic, marginal zinc deficiency. In the first case, an inverse relationship exists, whereas in the second case, a positive correlation is suggested. Thus, comparisons of group means is probably inadequate to clarify the relationship between these two indices.

In summary, the heterogeneity of the hair zinc concentrations in the group of small-for-age functionally delayed children, with undiagnosed cause of the delay suggests that their hair zinc concentration was not uniformly related to their poor growth. In several individuals, however, either low or relatively high hair zinc concentrations would warrant further assessment of their zinc nutriture to determine its relation to their growth pattern. The low hair zinc levels seen in several normal sized, apparently healthy children suggests that a number of such children may have marginal zinc nutriture or that hair zinc may be a reflection of metabolic needs.

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FETAL AND MATERNAL SERUM COPPER AND ZINC LEVELS IN HUMAN PREGNANCY

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ABSTRACT

Copper and zinc levels were measured in maternal mid-pregnancy (18-week and 32-week) and umbilical cord sera samples taken from approximately 1,000 women in South Australia. The data were related to pregnancy outcome, and to the ethnic background and certain characteristics of the mothers.

Copper levels rose in the maternal serum during gestation while zinc levels were lower in pregnant than in non-pregnant women. Cord serum copper was lower than in the maternal samples but cord zinc was higher.

Neither maternal weight, age nor parity appeared to exert a major effect on thelevels of either element, but clinically-assessed maternal obesity was associated with elevated serum copper levels.

Maternal serum copper and zinc levels were unrelated to the length of gestation or to fetal birthweight, although superficially there appeared to be a negative correlation. This observation is discussed in the light of recent, similar findings by other workers concerning maternal zinc status and infant birthweight.

KEY WORDS: Copper, zinc, fetal, maternal, pregnancy.

INTRODUCTION

The importance of trace elements during fetal development is now widely recognized, and copper and zinc in particular have received considerable attention (1). Both metals occur as prosthetic groups in a variety of metalloenzymes, and in a number of species a maternal deficit of either element during pregnancy has been demonstrated to be highly teratogenic (2).

In man, apart from the genetic copper deficiency associated with Menke's kinky hair syndrome, an *in utero* copper deficit appears to be unlikely (3) and little attention has been paid to maternal copper status and pregnancy outcome. On the other hand, the possibility of zinc impoverishment during pregnancy is generally acknowledged (4), and a link has been suggested between sub-optimal zinc status and congenital defects in the Middle East (5), Turkey (6), Sweden

(7), West Germany (8), and in the United States of America in association with dietary insufficiency (9) and in connection with the genetic zinc deficiency disease acrodermatitis enteropathica (10).

Since fetal copper and zinc derives from the blood of the mother, the level of these elements in the maternal plasma during pregnancy would be expected to be of critical importance to fetal nutrition. To date, most evidence suggests that plasma copper levels rise during pregnancy (11,12) and that zinc levels fall (11-13), although contrary trends have been reported with respect to zinc (12). The mechanisms underlying these pregnancy-induced changes are not understood, but the rise in serum copper is though to be due to estrogen-induced synthesis of ceruloplasmin, and the fall in zinc due to altered binding affinity of zinc-binding proteins (13,14), and to zinc uptake by the fetus and other products of conception.

Because of the importance of maternal copper and zinc for fetal development and since little is known concerning the copper and zinc status of Australian women, the present study was undertaken to examine the serum copper and zinc levels in a group of approximately 1,000 pregnant women in South Australia at two stages during pregnancy. In addition, umbilical cord blood samples were taken at delivery. Certain sociodemographic data were obtained which allowed some evaluation to be made of the factors which might affect maternal trace element status. In addition, a variety of indices were recorded concerning the outcome of pregnancy in the study subjects, which enabled possible associations to be examined between the levels of copper and zinc in the mother and fetus and the length of gestation, infant birthweight and placental weight at parturition.

METHODS

During the calendar year 1979, 1,150 out of 1,283 consecutive pregnant women attending the antenatal clinic at the Queen Victoria Hospital, Adelaide, South Australia, were enrolled into the study. The 133 (10%) not involved comprised miscarriages, antenatal care cancellations and women transferred to other hospitals. Twins were also excluded from the study.

These women were part of an ongoing, longer-term clinical epidemiological surveillance project. Hence, detailed information on antenatal care, personal, behavioural and sociodemographic characteristics, delivery, and neonatal assessment was collected in standardised fashion from clinical records and from individual interview. In addition the residues of three blood samples were obtained which were an alpha-fetoprotein (AFP) screening program maternal blood sample, taken at 16-19 weeks (18-week sample), and obtained from the Adelaide Children's Hospital, which co-ordinated the screening program; a sample taken at 32 weeks for antibody determination; and an umbilical cord blood sample. The latter two samples were sought directly from the Queen Victoria Hospital.

Since the blood samples were not being taken primarily for the purposes of this research project, some were omitted (especially at 32 weeks). Also all samples showing signs of hemolysis were discarded from the study. However, missing samples were unlikely to be biased with respect to the relationships being studied here and the numbers of satisfactory samples available for trace element determination were:

AFP, 18-week sample:	786
32-week sample:	475
Cord sample:	756

Of the original (1150) study group, a subset of 878 women had additional information collected on smoking, drinking and dietary habits as well as extra birth outcome information, which concerned the actual details of birthweight, gestation, mother's age and placental weight.

Blood samples were collected into disposable plastic syringes (Terumo Australia Pty. Ltd., Melbourne, Australia) and vials, which had been previously checked to be free from contamination by copper and zinc. All glassware used in laboratory procedures was acid-washed in dilute nitric acid.

Zinc and copper analyses were performed on serum samples diluted 10-fold with distilled water, and aspirated directly into a Perkin-Elmer Model 5000 atomic absorption spectrophotometer.

Mean levels of serum zinc and copper at 18 weeks, 32 weeks and in cord blood were calculated for various maternal variables and birth outcomes. The means, standard errors and numbers are presented for relevant categories of these variables. The F-test (one-way analysis of variance) was used to test for significant variations in the mean copper and zinc level among the categories of the variables under study (i.e. gestation, birthweight etc.). To test specific pairs of group means for differences, the least significant difference (LSD) test was used, in this case including the correction for samples of unequal size (15). A multivariate approach was used to examine the relationship of trace element level to the outcomes birthweight and gestation, controlling for other confounding variables.

RESULTS AND DISCUSSION

Copper and Zinc Levels in Maternal (18- and 32-week) and Fetal Serum

A comparison of the copper and zinc levels in maternal and cord blood sera against mean values for a normal, non-pregnant, young adult female population (Table 1) indicated that serum copper levels in the South Australian women rose during pregnancy ($p<0.0005$) to a point similar to that reported by other workers (11,12,16), although in the South Australian women the initial rate of increase appeared to be greater.

Maternal serum zinc levels were significantly reduced ($p<0.005$) when compared with the non-pregnant population mean (Table 1), which is in agreement with several other workers studying entirely different population groups (11-13). However, in the present study the fall in serum zinc occurred predominantly in the first 18 weeks, and did not increase with the progression of pregnancy as has been reported in some cases (17).

Fetal (umbilical cord) sera reflected copper levels approximately one quarter of those in maternal serum at 32 weeks, which is in accord with data from other workers (16,17). Cord blood serum zinc was appreciably (27%) higher than in maternal sera, which too is consistent with other reports (12).

The relative concentrations of maternal (32 week) and fetal (cord) serum zinc and copper showed clear differences. While cord blood has higher zinc levels it contains markedly less copper. The copper:zinc ratio varied from 2.9 in the 32-week pregnant woman to 0.5 in the fetus at delivery. The reason for these differences is not clear, although it has been suggested that the fraction of "free" copper and zinc in maternal and fetal blood is more similar, and is not influenced by the concentration and affinity of metal-binding proteins on either side of the placental barrier, as probably occurs with the "bound" elements (18).

For each element positive correlations were observed between levels at 18 weeks and subsequently at 32 weeks of pregnancy (Table 2). With copper, a rather less obvious positive correlation was also noted between maternal serum and cord blood, but with zinc a negative relationship was observed between 32-week maternal samples and cord sera.

TABLE 1

Copper and Zinc Levels in Maternal and Fetal Serum

Sample	Copper[a] (µg/ml)		Zinc[a] (µg/ml)	
Non-pregnant[b]	1.21±0.07	(25)	0.86±0.03	(25)
18-week	2.04±0.01[c]	(786)	0.73±0.01[e]	(786)
32-week	2.19±0.02[c,d]	(475)	0.73±0.01[e]	(475)
Cord	0.49±0.01	(756)	1.01±0.01	(756)

[a]Means±SEM. Numbers of subjects given in parentheses.
[b]Healthy non pregnant women, aged 20-30 years.
[c]$p<0.0005$ (t-test) compared with non-pregnant serum.
[d]$p<0.0005$ (t-test) compared with 18-week serum.
[e]$p<0.005$ (t-test) compared with non-pregnant serum.

TABLE 2

Spearman Rank Correlation Coefficients (R) within Elements between Sample Groups

Element	Sample Group	32-week (R)	Cord (R)
Copper	18-week	0.53[b] (218)	0.11[c] (468)
	32-week	-	0.15[d] (347)
Zinc	18-week	0.23[b] (279)	0.07[e] (469)
	32-week	-	-0.13[c] (347)

[a]Number of samples given in parentheses.
[b]$p<0.001$ compared with 32-week serum.
[c]$p<0.01$ compared with with cord serum.
[d]$p<0.005$ compared with cord serum.
[e]Not significant

Effect of Maternal Weight on Serum Copper and Zinc Levels

Serum copper and zinc levels were examined in groups of women segregated on the basis of maternal weight and classified either as under-weight, normal or obese in terms of their weight:height ratio as coded by an obstetrician (G.T.G.) on their first antenatal visit. Serum copper was significantly higher in the obese group than in the other categories at both stages during pregnancy (Table 3). For copper (18-week) the LSD-test showed that the obese group was significantly different from mothers having normal and underweight children ($0<0.01$). For copper (32-week), the obese group was again significantly different from mothers with normal weight children ($p<0.05$). At present the mechanism underlying this effect is not clear, nor is it known whether the rise is accompanied by a corresponding increase in ceruloplasmin levels.

Maternal serum zinc levels appeared to be unaffected by the weight of the mother during pregnancy, as also were cord blood sera with respect to both elements.

TABLE 3

Serum Copper and Zinc Levels in Relation to Maternal Weight

Sample	Copper[a] (µg/ml)	Zinc[a] (µg/ml)
18-week		
Under-weight	1.99±0.04 (70)	0.74±0.01 (71)
Normal	2.00±0.02 (522)	0.73±0.01 (523)
Obese	2.16±0.03 (194)	0.73±0.02 (194)
	*p<0.001	
32-week		
Under-weight	2.20±0.05 (40)	0.69±0.02 (40)
Normal	2.15±0.02 (302)	0.74±0.01 (303)
Obese	2.27±0.03 (133)	0.72±0.01 (133)
	*p<0.007	
Cord		
Under-weight	0.50±0.03 (57)	0.98±0.02 (57)
Normal	0.48±0.01 (510)	1.01±0.01 (510)
Obese	0.51±0.01 (189)	1.03±0.01 (189)

[a] Means±S.E.M. Numbers of subjects given in parentheses.
* ANOVA - F-test significance level

Effect of Maternal Age and Parity on Serum Copper and Zinc Levels

In both 18-week and 32-week samples, maternal age had little effect on copper and zinc levels, although copper levels appeared to decline in the oldest age group (Table 4). Neither element was affected in cord blood.

TABLE 4
Serum Copper and Zinc Levels in Relation to Maternal Age

Sample		Copper[a] (µg/ml)	Zinc[a] (µg/ml)
18-week			
Age (years)	<20	1.96±0.02 (155)	0.74±0.01 (156)
	20-29	2.05±0.02 (503)	0.73±0.01 (503)
	30-34	2.09±0.04 (96)	0.71±0.01 (97)
	35-39	2.09±0.07 (25)	0.72±0.02 (25)
	40+	1.85±0.05 (5)	0.74±0.03 (5)
32-week			
Age (years)	<20	2.19±0.04 (99)	0.73±0.01 (99)
	20-29	2.19±0.02 (304)	0.73±0.01 (310)
	30-34	2.23±0.06 (49)	0.74±0.02 (49)
	35-39	2.15±0.11 (13)	0.69±0.02 (13)
	40+	1.93±0.10 (4)	0.71±0.04 (4)
Cord			
Age (years)	<20	0.51±0.01 (143)	1.03±0.01 (143)
	20-29	0.48±0.01 (483)	1.01±0.01 (483)
	30-34	0.47±0.02 (98)	1.01±0.01 (98)
	35-39	0.51±0.03 (23)	1.06±0.04 (23)
	40+	0.44±0.06 (6)	0.99±0.05 (6)

[a] Means±S.E.M. Number of subjects given in parentheses.

Because age and parity are highly correlated, a similar pattern would be expected to be associated with the number of children. In order to control for the effect of age, parity was studied only within the age group 20-29 years.

The number of children previously borne by the women under study had little influence on the serum copper or zinc levels in both maternal and cord blood samples (Table 5). However, some suggestion was evident of a reduction in maternal zinc levels at 32 weeks in mothers with two or more previous children. Statistically significant differences were observed in the 18-week and cord blood copper analyses, but the trends were not linear and interpretation of the data is unclear.

TABLE 5
Serum Copper and Zinc Levels in Relation to Number of Children for Women Aged 20-29 Years

Sample		Copper[a] (µg/ml)	Zinc[a] (µg/ml)
18-week			
No.children	0	1.97±0.02 (289)	0.74±0.01 (289)
	1	2.11±0.03 (122)	0.72±0.01 (123)
	2	2.07±0.07 (37)	0.78±0.03 (37)
	3+	1.94±0.13 (8)	0.80±0.04 (8)
		*p<0.003	
32-week			
No.children	0	2.16±0.03 (188)	0.73±0.01 (188)
	1	2.28±0.04 (83)	0.73±0.01 (83)
	2	2.27±0.09 (27)	0.69±0.02 (27)
	3+	2.40±0.32 (4)	0.68±0.05 (4)
Cord			
No.children	0	0.51±0.01 (269)	1.02±0.01 (269)
	1	0.48±0.01 (109)	1.00±0.02 (109)
	2	0.43±0.02 (31)	0.96±0.02 (31)
	3+	0.55±0.06 (5)	1.00±0.06 (5)
		*p<0.01	

[a] Means±S.E.M. Numbers of subjects given in parentheses.
* ANOVA F-test significance level.

Effect of Ethnic Background on Serum Copper and Zinc Levels

Most groups studied demonstrated a tendency for maternal serum zinc levels to remain fairly constant between 18 and 32 weeks of pregnancy (Table 6). Copper levels generally rose continuously throughout the study period. By contrast Aboriginal serum zinc levels, which at 18 weeks registered the lowest value of any group, rose by 32 weeks to be the highest level recorded. Copper levels, too, rose proportionately more than in any other group.

In blood samples from women of Asiatic origin, both serum zinc and especially serum copper levels at 32 weeks appeared to be lower than average.

In most groups, zinc levels were higher in cord blood, reflecting the tendency for zinc to be accumulated transplacentally into the fetus. Conversely, copper levels were reduced in cord sera. Again, samples of Aboriginal origin reflected a different trend, and cord serum zinc levels were no higher than in the maternal blood at 32 weeks. However cord serum copper levels were above average, and thus were consistent with the unusually high 32-week maternal blood copper values.

Unfortunately, the data relating to Aboriginals were obtained from only three blood samples in each group, which makes it impossible to draw definite conclusions concerning the apparent differences. Nevertheless, these preliminary observations raise the question whether transplacental transfer of trace metals, especially zinc, may be different in this group. Certainly the suggestion has been made that a variant form of the zinc metalloenzyme carbonic anhydrase occurs as an adaptive mechanism in Aboriginals (19) and it may be that further genetic differences in zinc metabolism also exist. The singularly low level of zinc in the 18-week samples is of interest in the light of the depressed zinc status reported in Aboriginals by other workers (20). An expanded investigation into this question is now under way.

TABLE 6
Serum Copper and Zinc Levels in Relation to Ethnic Background

Group	Copper[a] 18 wk		32 wk		Cord	
Australian and New Zealand	2.03(±0.01)	(580)	2.19±0.03	(333)	0.49±0.01	(537)
Australian Aborigine	2.04(±0.26)	(3)	2.81±0.09	(3)	0.54±0.02	(3)
British	2.05(±0.03)	(97)	2.09±0.04	(55)	0.48±0.02	(91)
Italian	2.13(±0.07)	(30)	2.31±0.08	(28)	0.54±0.03	(34)
Greek	2.09(±0.08)	(16)	2.21±0.14	(10)	0.46±0.04	(22)
Yugoslavian	1.78(±0.08)	(6)	2.28±0.10	(7)	0.40±0.04	(8)
Other European	2.12(±0.05)	(29)	2.24±0.11	(17)	0.52±0.03	(32)
Asian	2.04(±0.08)	(8)	1.97±0.11	(5)	0.44±0.04	(8)
Other	1.87(±0.09)	(15)	2.14±0.07	(13)	0.41±0.02	(17)

Table 6 (cont'd)

Group	Zinc[a] 18 wk		32 wk		Cord	
Australian and New Zealand	0.73±0.01	(581)	0.73±0.01	(334)	1.02±0.07	(537)
Australian Aborigine	0.64±0.03	(3)	0.83±0.09	(3)	0.80±0.06	(3)
British	0.74±0.01	(98)	0.73±0.02	(55)	1.01±0.02	(91)
Italian	0.75±0.02	(30)	0.71±0.02	(28)	0.98±0.02	(34)
Greek	0.71±0.03	(16)	0.70±0.04	(10)	1.00±0.04	(22)
Yugoslavian	0.76±0.04	(6)	0.70±0.03	(7)	0.99±0.04	(8)
Other European	0.70±0.02	(29)	0.76±0.03	(17)	1.08±0.03	(32)
Asian	0.69±0.03	(8)	0.71±0.02	(5)	0.95±0.05	(8)
Other	0.71±0.02	(15)	0.78±0.03	(13)	0.96±0.04	(17)

[a]Means±S.E.M. Numbers of subjects are indicated in parentheses.

Copper and Zinc Levels in Relation to Length of Gestation and to Birthweight

Mid-pregnancy serum samples displayed evidence of a weak inverse relationship to the length of gestation, which suggested that postmaturity may be associated with lower serum copper and zinc levels. However the trend failed to attain statistical significance (Table 7).

TABLE 7
Serum Copper and Zinc Levels in Relation to Length of Gestation

Sample	Gestation (weeks)	Copper[a] (µg/ml)		Zinc[a] (µg/ml)	
18-week					
	< 37	2.11±0.04	(54)	0.76±0.02	(54)
	37-38	2.05±0.04	(110)	0.73±0.01	(110)
	39-40	2.03±0.02	(512)	0.73±0.01	(514)
	> 40	2.02±0.03	(110)	0.72±0.01	(110)
32-week					
	< 37	2.25±0.08	(20)	0.73±0.03	(20)
	37-38	2.22±0.05	(58)	0.72±0.01	(58)
	39-40	2.21±0.02	(334)	0.73±0.01	(335)
	> 40	2.07±0.04	(63)	0.71±0.01	(63)
Cord					
	< 37	0.43±0.06	(25)	1.01±0.04	(25)
	37-38	0.45±0.01	(101)	1.02±0.02	(101)
	39-40	0.49±0.01	(511)	1.01±0.01	(511)
	> 40	0.54±0.02	(119)	1.03±0.01	(119)
		*p<0.001			

[a] Means±S.E.M. Numbers of subjects given in parentheses.
* ANOVA F-test significance level.

Since, in general, birthweight is positively correlated with duration of gestation, a possible inverse relationship between mid-pregnancy maternal serum copper and zinc status, and duration of gestation would be expected to be mirrored in a similar relationship between the maternal trace element status and birthweight. Such a negative association is weakly suggested in zinc levels at 18 weeks and with both elements at 32 weeks, although the trend is not statistically significant and is not mirrored in the 18-week copper levels or in cord blood (Table 8).

TABLE 8
Serum Copper and Zinc Levels in Relation to Birthweight

Sample	Birthweight (kg)	Copper[a] (µg/ml)	Zinc[a] (µg/ml)
18-week			
	< 2.5	2.00±0.04 (63)	0.76±0.02 (63)
	2.5-3.0	2.07±0.03 (131)	0.75±0.01 (131)
	3.0-3.5	2.00±0.02 (285)	0.73±0.01 (286)
	3.5-4.0	2.04±0.02 (231)	0.73±0.01 (231)
	> 4.0	2.09±0.03 (76)	0.72±0.01 (77)
32-week			
	< 2.5	2.25±0.09 (19)	0.77±0.03 (19)
	2.5-3.0	2.27±0.40 (83)	0.72±0.01 (83)
	3.0-3.5	2.19±0.03 (168)	0.71±0.01 (168)
	3.5-4.0	2.15±0.03 (159)	0.74±0.01 (160)
	> 4.0	2.16±0.05 (46)	0.72±0.03 (46)
Cord			
	< 2.5	0.50±0.03 (31)	1.04±0.04 (31)
	2.5-3.0	0.51±0.20 (115)	1.02±0.01 (115)
	3.0-3.5	0.47±0.01 (283)	1.02±0.01 (283)
	3.5-4.0	0.49±0.01 (252)	1.00±0.01 (252)
	> 4.0	0.50±0.02 (75)	1.00±0.02 (75)

[a]Means±S.E.M. Number of subjects given in parentheses.

Since both birthweight and length of gestation have been studied extensively, and are known to be influenced by other factors such as maternal age and smoking habits, multiple regression analyses were carried out in order to estimate the true independent effects of maternal serum zinc and copper levels upon each of these two pregnancy outcomes. Independent variables included in the multivariate model were: zinc and copper levels at the 18th week and the 32nd week, maternal age, gravidity, weight, cigarette smoking, and, either, birthweight or gestation (depending on which of these was the outcome variable).

For birthweight, zinc and copper levels at 18 weeks add nothing to the explained variance (R^2) of birthweight in the best-fitting multiple linear regression model and therefore play a minor role as birthweight determinants. Copper levels at the 32nd week display borderline significance when added into the equation ($p<0.07$), but add only $R^2 = 0.01$ to the total equation ($R^2 = 0.17$). Zinc levels add nothing. With gestation as the outcome variable, only copper levels have some significance ($p = 0.06$), although the relationship is weak. Thus, overall, neither maternal serum zinc nor serum copper levels appear to make a substantial contribution to either outcome of pregnancy.

Thus, despite considerable evidence from animal studies of an association between reduced birthweight and mid-pregnancy maternal zinc deficiency, the present study and those of Jameson (7), Metcoff et al. (21) and Cherry et al.

(22) have found no such association in humans. Indeed, the first two workers reported that below-average maternal zinc status was associated with increased birthweight, with or without prolonged gestation and postmaturity - a trend weakly reflected in the present study. These contrasting observations have already provoked comment and the possibility has been raised by Metcoff et al. (23) in general terms, and by Metcoff (21) and McMichael et al.(24) specifically in relation to zinc, that in some cases above-average placental and fetal uptake of zinc may tend to produce below-average maternal serum zinc concentrations in generally well-nourished populations. Thus, while on the one hand diminished mid-pregnancy serum zinc levels might indeed denote a maternal micronutrient deficiency, as would be expected in controlled animal experiments and in poorly-nourished human communities, similar values under other circumstances may arise from more efficient placental transfer of the nutrient from mother to fetus, or from more active uptake of the available element by the fetus. Prema (25), too, has noted an association between low copper/high zinc levels in mid-pregnancy serum samples from Indian women and the occurrence of small birthweight infants. He considers that these differences may be due in part to intrauterine growth retardation. Possibly, in questionable cases, concurrent use of an alternative index of maternal zinc status (e.g. hair zinc levels) may provide information which would enable a distinction to be drawn between these two situations.

No association was demonstrated between cord blood zinc and copper levels and birthweight (Table 8), but a strong positive correlation seems to exist between cord blood copper levels and postmaturity (Table 7). Possibly, this relationship reflects the active accumulation of copper that occurs in the fetus at the end of pregnancy (26). It is therefore quite likely that the reduced maternal copper levels associated with prolonged gestation and the increased cord blood concentrations together arise as a consequence of the active fetal copper metabolism which occurs immediately prepartum.

Copper and Zinc Levels in Relation to Placental Weight

The maternal samples at 18 weeks indicated a possible inverse relationship between serum zinc levels and placental weight at birth (Table 9). Serum copper levels at 32 weeks were similarly negatively correlated. Cord sera by contrast exhibited a weak positive trend with respect to zinc but nothing definite in relation to copper.

TABLE 9
Serum Copper and Zinc Levels in Relation to Placental Weight

Sample	Placental wt. (g)	Copper[a] (µg/ml)	Zinc[a] (µg/ml)
18-week			
	< 400	2.03±0.05 (41)	0.77±0.02 (41)
	- 450	2.03±0.05 (53)	0.74±0.02 (53)
	- 550	2.02±0.02 (255)	0.73±0.01 (255)
	- 600	2.08±0.03 (131)	0.73±0.01 (132)
	- 700	2.02±0.03 (186)	0.73±0.01 (187)
	700+	2.03±0.03 (111)	0.72±0.01 (111)
32-week			
	< 400	2.34±0.09 (20)	0.73±0.02 (20)
	- 450	2.25±0.08 (41)	0.75±0.02 (41)
	- 550	2.22±0.03 (150)	0.72±0.01 (150)
	- 600	2.18±0.05 (72)	0.74±0.01 (72)
	- 700	2.16±0.03 (119)	0.75±0.02 (120)
	700+	2.12±0.04 (68)	0.69±0.02 (68)
Cord			
	< 400	0.54±0.03 (25)	0.97±0.03 (25)
	- 450	0.49±0.02 (51)	0.99±0.03 (51)
	- 550	0.48±0.01 (242)	1.02±0.01 (242)
	- 600	0.48±0.01 (122)	0.97±0.01 (122)
	- 750	0.48±0.01 (188)	1.02±0.01 (188)
	700+	0.51±0.02 (121)	1.03±0.01 (121)

[a] Means±S.E.M.

CONCLUSIONS

Overall, the present study on South Australian women confirms the upward trend in maternal serum copper levels and the decline in zinc concentrations reported for other populations of pregnant women. Apart from the effect of maternal obesity on serum copper levels, no significant associations with maternal trace element status were found for maternal age, weight and parity. Nor were the maternal serum copper and zinc levels related to the length of gestation or to birthweight, which is different from experimental data and has received some discussion. Limited observations on Aboriginal women suggest that this group in particular warrants further study. No reference is made in the present paper to maternal trace element status in relation to complications associated with pregnancy or to fetal teratogenesis, as these form the basis of a separate study.

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THE EFFECTS OF MILK PROTEIN FEEDING ON SPECIFIC INTESTINAL PROTEIN PERMEABILITY IN THE GUINEA-PIG

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ABSTRACT

Dietary bovine milk protein induced suppresssion of milk protein transport across the small intestine in guinea-pigs. The degree of suppression was greater in animals fed from neonatal age to adulthood compared to the suppression induced in adult animals fed for the same period. Serum antibody responses to dietary milk protein were similar for the two feeding regimes and showed a predominant IgG2 response. *In vitro* measurements of gut anaphylaxis have shown that neonatally fed animals fail to respond to milk protein challenge, in contrast to animals fed milk protein as adults.

KEY WORDS: Milk protein, intestinal permeability/transport, antibody, anaphylaxis.

INTRODUCTION

Administration of antigenic material to animals via the gastrointestinal tract induces lymphoid responses that vary from immunity to hyporesponsiveness (1). It is evident that some of the material reaching the gastrointestinal tract escapes digestion and reaches the lymphoid system. *In vitro* analysis of intestinal permeability be everted sac assay (2) has shown that dietary protein can cross from the mucosal to serosal compartment (3). Feeding laboratory animals radiolabelled protein and measuring radioactivity in the mesenteric circulation has demonstrated that up to 2% of the ingested dose may reach the circulation (4). The permeability of mammalian small intestine may be greatest or least specific during neonatal life (5). Antibodies of the major immunoglobulin classes were detected in the serum of perorally immunised neonatal pigs (6). Various reports have suggested that pre-feeding laboratory animals reduces or abrogates antibody mediated anaphylactic responses to the pre-fed antigens (7,8).

In the present report we have examined the influence of bovine milk feeding upon intestinal permeability in neonatally and adult fed guinea-pigs together with serum antibody responses and *in vitro* anaphylaxis.

MATERIALS AND METHODS

Animals. Outbred Hartley strain guinea-pigs of either sex were used throughout this study. Animals were supplied by A. Tuck and Son, Essex, as

young adults weighing between 180 g and 220 g. Pregnant female guinea-pigs were supplied and the litters delivered after the transit period. Young animals were tested after a three month experimental feeding schedule at weights that varied between 280 g and 350 g.

FEEDING SCHEDULE

Feeding schedule. A milk protein-free solid pellet diet was supplied to all animals. Guinea-pigs were supplied, as cages of three, with 350 cm^3 of 50% (v/v) bovine milk in water each day for three months. Neonatal guinea-pigs were weaned at ten days and placed under the milk and solid diet described above for three months. The weights of milk-weaned animals at three months varied between 250 g and 300 g. Some experiments were carried out on tissues from adult guinea-pigs fed with solid diet plus bovine β-lactoglobulin supplemented water supplied daily over a twenty-eight day period (1% w/v β-lactoglobulin in distilled water).

Sensitising schedules. Adult guinea-pigs (380 g), not under milk diet, were parenterally sensitised with β-lactoglobulin in Freunds Complete Adjuvant as described for bovine gamma globulin (9).

Iodination of protein. Bovine β-lactoglobulin and chicken ovalbumin were iodinated with sodium iodide-125 using the iodine monochloride method (10). Iodination was performed to substitute approximately one atom of iodine per molecule of protein. Separation of protein-bound from free iodine was achieved by gel filtration of Sephadex G25.

Everted sac assay. Animals were starved for twenty-four hours prior to assay. The small intestine from a freshly sacrificed guinea-pig was cannulated and 40 cm^3 of saline (5°C) was injected into the intestine before removing the ileal section and placing this in a trough containing saline. Sections of ileum (10 cm) were everted using glass rods and secured following the incision of 1 cm^3 oxygenated Krebs-bicarbonate solution. Sacs were incubated at 37°C in 20 cm^3 Krebs-bicarbonate containing between 300 and 800 ug 125-I-protein for sixty minutes. Samples (0.2 cm^3) were removed from the sacs and assayed for radioactivity using a Phillips PW 4580 gamma counter. All data presented is the radioactivity that was acid precipitable, i.e. protein bound.

Haemagglutination assay. Guinea-pig erythrocytes have been used in all assays, these were obtained from animals not under milk feeding regimes. Proteins were conjugated by the chromic chloride method (11). Volumes of 2% (v/v) protein conjugated erythrocytes were mixed with equal volumes of serially diluted serum from either milk fed or control animals. Titres were recorded after incubation of the reactants at room temperature for two hours.

Antiglobulin analysis was carried out by titrating erythrocyte-antigen conjugates sensitised with dilutions of subagglutinating guinea-pig serum from milk fed animals with optimal dilutions of antiglobulin reagent (anti guinea-pig immunoglobulin sera). Assays were performed as described in (12). Sera for analysis was sera from cages of three animals, the sera pooled prior to assay. Each assay represents the analysis from at least three cages of animals.

Anaphylactic responses. Schultz/Dale assays were performed on ileal tissue from animals under the various dieting regimes. Responses were monitored using a Bioscience UF1 transducer Washington FC137 recording system.

Antisera. Monospecific rabbit anti guinea pig IgG2 and IgG1 was prepared as described earlier (9). Monospecific sheep anti guinea-pig IgM and rabbit anti guinea-pig IgA were obtained from Miles Laboratories.

RESULTS

Everted sac assay. The permeability of 10 cm sections of guinea-pig ileum to radiolabelled macromolecules, as measured by the everted sac technique, is different in 24 hours neonates to the values obtained when adult tissues are tested. In Table 1 it may be seen that 1.76% of the ^{125}I-β-lactoglobulin added to the mucosal compartment is detectable in the serosal compartment when 24 hour neonatal intestine is used. In contrast, values of between 0.5% and 0.6% are obtained when tissues from normal adults are assayed.

Experiments measuring the rate of uptake of ^{14}C-glycine by guinea-pig ileal sacs during a 60 minute period consistently yielded a tissue : medium ratio of radiolabelled amino acid in excess of 1.0 (B.M. Carrick, unpublished findings). These findings indicate that active transport mechanisms remain operative under such conditions and are a measure of tissue viability. Ileal tissue incubated under anoxic conditions for 24 hours did not manifest tissue accumulation of radiolabelled glycine. Such tissues showed non-selective high permeability towards the two proteins studied.

Introducing adult guinea-pigs to a bovine milk diet for three months specifically affects the permeability of ^{125}I-β-lactoglobulin. The percentage of ^{125}I-β-lactoglobulin recorded in the serosal compartment was 0.38 ± 0.05% contrasted with 0.52 ± 0.06% obtained in tissue from control animals. As can be seen in Table 1, the permeability of ^{125}I-ovalbumin is unaffected by the milk diet.

A greater difference in intentinal permeability was obtained from analysis of tissue from guinea-pigs weaned on to bovine milk at ten days and maintained on a milk diet for three months. The permeability to ^{125}I-β-lactoglobulin is reduced to 0.24 ± 0.041%, a reduction of approximately 50% in milk protein permeability compared to control values of 0.5 ± 0.06%. As in the adult fed groups, permeability to ^{125}I-ovalbumin was similarly unaffected.

Parenteral sensitisation to bovine β-lactoglobulin also specifically influenced the intestinal permeability to ^{125}I-β-lactoglobulin, reducing it to values similar to those obtained from adult milk fed animals, or those fed β-lactoglobulin supplemented water.

Serum agglutinating antibody to milk proteins was measured in terms of antibody titre and antibody class. Low titres to β-lactoglobulin are detectable at four days in adults following the introduction to dietary milk (Table 2). Antibodies to casein and bovine albumin appeared later. The titre of antibodies to β-lactoglobulin was 1024 on day 15 and between 1024 and 8192 at the end of the feeding schedule. Antibody titres in the serum of weaned animals were lower than those obtained for the adult fed group (Table 2).

In all animals, whether adult fed or weaned, the major serum antibody response was in the IgG2 subclass, with lower serum titres to IgG1, IgM and IgA (Table 3).

TABLE 1

Mucosal to Serosal Permeability Measurements from Ileal Tissue of Guinea-Pigs Under Various Dietary Regimes

	Regime	Age at Assay	(a) % ^{125}I-β-lacto-globulin added recovered in intestinal serosal compartment[a]	(b) % ^{125}I-Oval-bumin added recovered in intestinal serosal compartment[a]
I.	Maternal milk	24 hrs	1.76 ± 0.11[b]	N.D.
II.	Solid diet/water (3 mths)	4 mths	0.52 ± 0.06	0.50 ± 0.061
III.	Solid diet/bovine milk (3 mths)	4 mths	0.38 ± 0.05	0.06 ± 0.02
IV.	Solid diet/weaned on to bovine milk at 10 days old, for three months	4 mths	0.24 ± 0.04	0.60 ± 0.03
V.	Solid diet/β-lactoglobulin supplemented water for 28 days	4 mths	0.32 ± 0.05	N.D.
VI.	Solid diet/water parenteral sensi-tisation with β-lactoglobulin	4 mths	0.38 ± 0.09	0.65 ± 0.05

a Initial iodinated protein added to mucosal compartment in everted sac assay and varied between 15 and 40 ug/cm^3.

b Number tested = 6 for all groups

Student's test analysis of the significance of
II vs III $p = 0.01$
II vs IV $p = 0.001$

N.D. Not Determined

TABLE 2

Serum Agglutinating Antibody Titres Recorded from Pooled Sera of Animals

Day	β-lactoglobulin[c]	E. Casein	E. Albumen
Animals introduced to bovine milk as adults			
2	-	-	-
4	2[a]	-	-
6	2	4	-
9	8	4	-
10	32	8	8
13	128	8	8
15	1024	128	128
60	8192[b]	512	512
Animals weaned on to bovine milk at ten days and maintained under regime for three months			
35	32		
60	512		

[a] Reciprocal titre of serum antibody to milk proteins
[b] Several groups of cage analysis yielded varying titres at 60 days
[c] Protein conjugated guinea-pig erythrocytes

TABLE 3

Antiglobulin Analysis of Serum Antibody Responses of Guinea-Pigs Under Various Milk Protein Dietary Regimes

Regime	Titre of serum used to sensitise β-lactoglobulin conjugated erythrocytes	IgM	IgG1	IgG2	IgA
	(a)	(b)			
Adult milk protein fed - three months	2000	+ (8)	+ (8)	++ (128)	+ (8)
Adults weaned on to milk (bovine) at ten days and regime maintained for three months	256	+ (2)	+ (2)	++ (32)	+ (8)

(a) Optimal subagglutinating titre of guinea-pig serum
(b) Reciprocal titre of antiglobulin reagent that gave optimal titre where control erythrocytes gave negative results
\+ Positive - Agglutination in between 1 and 4 minutes
++ Strongly positive - Agglutination in 1 minute

Anaphylactic response. The influence of milk protein feeding on anaphylactic responses is shown in Table 4. It can be seen that whereas adult milk fed or parentally sensitised animals carry anaphylactically sensitised tissue, animals weaned on to milk at ten days and maintained on milk for three months yielded intestinal tissues that consistently failed to respond to antigen challenge.

TABLE 4

Anaphylactic Responses by Ileal Sections from Guinea-Pigs Under Dietary Regimes

Regime	Challenging Antigen	Ng histamine released per ug of antigen added
Adult animals fed solid diet/water	Milk	-
	β-lactoglobulin	-
	Ovalbumin	-
Adult animals introduced to milk/solid diet as adults	Milk	1.2
	β-lactoglobulin	2.2
	Ovalbumin	-
Animals weaned on to bovine milk and fed milk/ solid diet until adulthood	Milk	-
	β-lactoglobulin	-
	Ovalbumin	-

Six animals were tested in each experimental.

DISCUSSION

In the present report, using an *in vitro* measure of intestinal permeability differences have been shown between neonatal and adult tissues in their permeability to radiolabelled proteins. The small intestine of the neonatal guinea-pig undergoes anatomical changes during the first 48 hours of life, and these contribute to the differences in permeability shown in Table 1 between day old and adult intestinal tissues. Studies with ^{125}I-PVP (5) have shown that by 48 hours guinea-pig intestinal macromolecular permeability is extremely limited.

Varying estimates of intestinal permeability to macromolecules have been reported (13) and the values obtained from everted sac assays may not be directly applicable to in vivo tissue. However, the relative permeability values reported here are clearly relevant to the interpretation of immune responses to dietary proteins. In common with other authors we have demonstrated that prefeeding adult animals milk proteins reduces the intestinal permeability of those proteins in a specific manner (7,8). Introducing neonatal guinea-pigs, at ten days to bovine milk feeding and continuing the regime for three months has a more profound influence upon specific intestinal permeability of milk proteins. Such animals yield ileal sections with markedly reduced permeability, in fact

63 per cent of the values observed for animals introduced to bovine milk feeding as adult. The enhanced suppression of permeability in the weaned groups of guinea-pigs is presumably due to dietary proteins passing from the mucosal to serosal compartment during the period from weaning to adulthood in quantities sufficient to provoke a more effective mucosal immune response.

Although permeability to ^{125}I-PVP may diminish markedly at 48 hours of age, dietary proteins may display different permeability characteristics.

Reduction in the absorption of dietary antigens following feeding has been demonstrated in mice (14). IgA antibody synthesis locally in the gut associated lymphoid tissue is considered responsible for the reduction in permeability.

Comparison of the concentration of mucosal IgA between neonatal, weaned guinea-pigs and adult milk fed animals is under investigation in the present laboratory.

Serum antibody responses in terms of antibody titre and class have been monitored in the present work during the milk feeding schedule. The antibody responses in weaned and adult fed groups are essentially similar except, that lower titres were consistently recorded in sera from the weaned animals. In the two groups, the IgG2 subclass was the predominant antibody produced with lower titres recorded for IgG1, IgGM and IgA. In contrast, rats fed intragastrically with protein antigens have been shown to mount a predominant IgM serum antibody response (15). The authors noted that protein antigens administered orally to some animals evoked an IgG response. Thus the difference reported in the present work may reflect either species differences or differences in dietary protein administration.

We have demonstrated that guinea-pigs introduced to bovine milk as adults manifest anphylactically sensitised ileal tissue. In contrast, the weaned guinea-pigs consistently failed to show anaphylactic responses when their ileal tissues were assayed by the Schultz-Dale system. It is interesting that these animals also manifested an enhanced suppression of intestinal permeability to milk proteins. Other laboratories have studied refractory anaphylactic responses to milk proteins in the guinea-pig and have implicated IgG1 as the subclass mediating the effect (8).

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TRANSFER OF α-TOCOPHEROL TO MICROSOMES MEDIATED BY A PARTIALLY PURIFIED LIVER α-TOCOPHEROL BINDING PROTEIN.[1]

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ABSTRACT

A liver α-[^{3}H]-tocopherol binding protein was partially purified using ammonium sulfate fractionation, gel filtration and ion-exchange chromatography from the 100,000 x g liver supernatant of rats fed a low vitamin E diet for 3 months and administered 200 μCi of α-[^{3}H]-tocopherol. The labelled protein was used to study the transfer and binding of α-tocopherol to liver microsomes. In vitro incubations showed that the transfer of α-tocopherol was directly proportional to the amount of α-[^{3}H]-tocopherol binding protein and to the amount of microsomal protein present. Moreover, the transfer of α-tocopherol increased with the temperature of incubation. A 3.8 fold excess of unlabelled α-tocopherol (bound to the same protein) inhibited the transfer of labelled α-tocopherol by 62%. These data suggest that an α-tocopherol binding protein could play a role in the intracellular transfer of α-tocopherol.

KEY WORDS: Vitamin E, α-tocopherol, α-tocopherol binding protein

INTRODUCTION

α-Tocopherol, the most active form of vitamin E (1) is present in the membrane of intracellular organelles where it is thought to play an important role in protecting lipids against oxidation (2).

The mechanism by which α-tocopherol is transported to intracellular organelles is unknown. Recent studies in vivo (3) and in vitro (4-7) showed that rat liver cytoplasm contains a protein with a molecular weight of 30,000-34,000 that binds α-tocopherol with high affinity and specificity, although its function remains unknown. Some work suggests that the biological role of this protein is to transfer α-tocopherol between membranes. Mowri et al (6) showed that transfer of α-tocopherol from liposomes to mitochondria was enhanced by this protein. Murphy and Mavis (7) also showed that this protein was able to transfer the vitamin from liposomes to microsomes.

The present study was undertaken to explore factors that influence the transfer of α-tocopherol from a partially purified liver α-tocopherol binding protein (α-TBP) to microsomes.

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MATERIALS AND METHODS

Animals and diets. Male Wistar rats (Canadian Breeding Farms, St. Constant, Québec) were used in all the experiments and were fed for three months on a modified AIN-76-low vitamin E diet (8) as follows (w/w): Casein 20.0%, DL-Methionine 0.3%, Cornstarch 15.0%, Sucrose 50.0%, Cellulose 5%, Lard 5.0% (Substituting for corn oil), AIN mineral mixture 3.5%, AIN vitamin mixture (Vitamin E-free) 1.0% Choline bitartrate 0.2%; or a modified AIN-76-high vitamin E diet in which D-α-tocopherol acetate was added to the corn oil to give 0.73 g per kg of diet.

Preparation of microsomal fraction. Microsomes were isolated according to the procedure described by Kornbrust and Mavis (9) with small modifications as follows: three rats (low vitamin E diet) with a body weight averaging 397 ± 9 g (SD) were anesthetized with halothane (2% in oxygen) and their livers were perfused via the hepatic portal vein with chilled 0.9% NaCl. After perfusion, the livers were excised, weighed and chilled with ice-cold 5 mM Tris-maleate buffer, pH 7.4, containing 0.15 M KCl, 1 mM EDTA and homogenized in the same buffer using a Teflon-glass homogenizer. The pooled homogenate was centrifuged successively at 300, 1,600, 8,000 and 30,000 x g for 10-15 min at each speed, and each of the pellets was discarded. Microsomes were obtained from the 30,000 x g supernatant by centrifugation at 100,000 x g for 1 hr. The pellet was washed once by suspension in and resedimentation from buffered potassium chloride with no EDTA. The final microsomal fraction was suspended in 0.1 M potassium phosphate buffer, pH 7.4 at a concentration of 1 mg protein/ml and stored at -75°C.

Purification of labelled α-tocopherol binding protein (α-[^{3}H]-TBP). Two rats (average body weight: 386 g) kept on a low vitamin E diet were used in this experiment. Each rat was injected intraperitoneally with 200 μCi of D-α-[5-methyl-^{3}H]-tocopherol (SA: 19.7 Ci/mmol, Amersham Corp. Arlington Heights IL) dissolved in 1.5 ml of saline solution containing 0.1 ml of ethanol and 0.05 ml Tween 80. 24 hrs after dosing, the rats were anesthetized with halothane and the livers perfused as indicated above. After perfusion, the livers were excised, weighed and chilled in 0.25 M sucrose containing 10 mM Tris-HCl buffer at pH 7.4 and 1 mM EDTA. A 50% liver homogenate was prepared in the same medium using a Teflon-glass homogenizer. The homogenate was centrifuged at 4°C for 20 min at 15,000 x g. The supernatant was recentrifuged in a Beckman L5-75B ultracentrifuge at 4°C for 1 hr at 100,000 x g to obtain a clear supernatant. The liver supernatant was then fractionated using ammonium sulfate. The fraction that precipitated between 30 and 60% of saturation was applied to a Sephadex G-100 column. The conditions of chromatography were identical to those reported previously (3). The radioactive fraction that contained α-tocopherol and that eluted at a molecular weight of 32,000 was collected and concentrated at 75% ammonium sulfate saturation. The resulting pellet was resuspended in 0.02 M Tris HCl buffer, pH 7.5 and dialyzed overnight in the same buffer, followed by ion-exchange chromatography on a 2.6 x 23 cm column of DEAE-Sephacel (Pharmacia, Uppsala, Sweden). The column was washed with 3 volumes of the same buffer (15 ml/hr), and subsequently the sample was eluted with a linear gradient of 0 to 0.5 M NaCl. Only one radioactive peak eluted from the column and was associated with the bulk of α-tocopherol. The peak eluted at a concentration of 0.14 M NaCl. The fraction was collected and used without further treatment.

An identical purification of the protein was conducted from rats kept on a high-vitamin E diet, without the administration of labelled α-tocopherol. The purification steps were monitored by estimating α-tocopherol, as indicated below.

α-Tocopherol binding to microsomes. The binding of α-[^{3}H]-tocopherol to microsomes was performed according to the procedure described by Murphy and Mavis (10) as follows: A known concentration of α-[^{3}H]-TBP was added to microsomes suspended in 0.01 M potassium phosphate buffer, pH 7.4 and incubated at 37°C (unless otherwise indicated) in a shaking water bath. The final volume of the incubation medium was 1 ml. Following incubation the α-tocopherol bound to microsomes was separated from α-TBP by negative pressure filtration with 0.22 μm mixed cellulose acetate and nitrate filters (Millipore

Intertech, Inc. Bedford, Mass). The filters were pretreated for 18 hrs in unlabelled α-TBP to minimize adsorption of the protein to the filter. To quantitate the amount of α-tocopherol bound to microsomes, 0.5-0.7 ml of the suspension was applied to a filter. The filter was immediately washed with 15 ml cold 0.1M potassium phosphate buffer, pH 7.4, and placed into a scintillation vial containing 15 ml of Aquasol-2 (New England Nuclear, Boston, Mass). After 45 min, the scintillation vial was vortexed, and the radioactivity was measured to determine the amount of α-[^{3}H]-tocopherol bound to microsomes. The radioactivity of a 0.1-0.2 ml aliquot of the same suspension was also measured to determine the total α-[^{3}H]-tocopherol. Aliquots of 0.1-0.2 ml were used to estimate α-tocopherol at the end of the incubation period. These two measurements indicated that the purity of the α-[^{3}H]-tocopherol at the end of the incubation period was greater than 92%. The amount of α-[^{3}H]-TBP adsorbed to the filter in the absence of microsomes was 4 to 6% of the amount applied and equal to the amount with microsomes present in an incubated control (Time 0), indicating that α-[^{3}H]-TBP was not being trapped by the microsomal particles. The loss of microsomes during filtration was determined by prelabelling the microsomes: 200 mg of microsomal protein was labelled by incubation in the presence of α-[^{3}H]-TBP, as indicated above for 180 min. The labelled microsomes were separated from the free α-[^{3}H]-TBP by centrifugation at 100,000 x g followed by washing and resuspension in 0.1 M potassium phosphate buffer, pH 7.4. Aliquots of the labelled microsomes were filtered as usual and the percentage of the total radioactivity retained by the filters determined. The retention was 99.4 $\pm$ 0.3 (SD, n = 3).

Other analytical methods. Protein was estimated by the method of Bradford (11) using a protein assay kit (Bio-Rad Laboratories, Richmond, CA.). α-tocopherol was estimated using the high performance liquid chromatography (HPLC) method of Thompson and Hatina (12) as previously indicated (3).

RESULTS

Purification of labelled and unlabelled α-TBP. Ammonium sulfate fractionation followed by gel filtration and ion exchange chromatography produced a partially purified α-TBP. When α-[^{3}H]-tocopherol was injected prior to sacrifice, the final protein preparation had a specific activity equal to 0.518 mCi/mg, and 0.072 µg α-tocopherol and 600 µg of protein per ml of solution. A preparation of 45 ml was obtained. When α-TBP was purified using the same procedure from livers of rats kept on a high-vitamin E diet, the final preparation contained 0.284 µg α-tocopherol and 500 µg of protein per ml of solution. A more detailed method of purification will be published elsewhere.

α-tocopherol binding to microsomes. The time course of binding of α-tocopherol to liver microsomes in the presence of α-[^{3}H]-TBP is shown in Figure 1. Apparent equilibrium was reached at three hours under the conditions of incubation described in the figure legend. All subsequent incubations were carried out at this time. The amount of α-[^{3}H]-tocopherol bound to microsomes was directly proportional to the amount of labelled protein present in the incubation medium (Figure 2). Moreover, the amount of α-[^{3}H]-tocopherol bound to microsomes was also directly proportional to the amount of microsomal protein as can be seen in Figure 3. The transfer of the vitamin was dependent on the temperature of incubation. At 0°C the transfer was completely inhibited but increased linearly as the temperature was raised (Figure 4). Incubation at 56°C results in no transfer due to destruction of α-tocopherol at this temperature. Figure 5 shows that increased amounts of unlabelled α-Tocopherol added as α-TBP were able to compete with the transfer of the labelled vitamin to microsomes. Increasing unlabelled tocopherol by 3.8 fold resulted in an inhibition equal to 62%.

FIG. 1

Time course of α-tocopherol binding to liver microsomes. Microsomes were incubated at 37°C in 1 ml of a medium containing 100 µg of microsomal protein and 83.5 nM of α-[^{3}H] tocopherol as α-[^{3}H] TBP (0.223 µCi/nmol) in 0.1 M potassium phosphate buffer, pH 7.4. Each point represents the mean ± SD of three determinations.

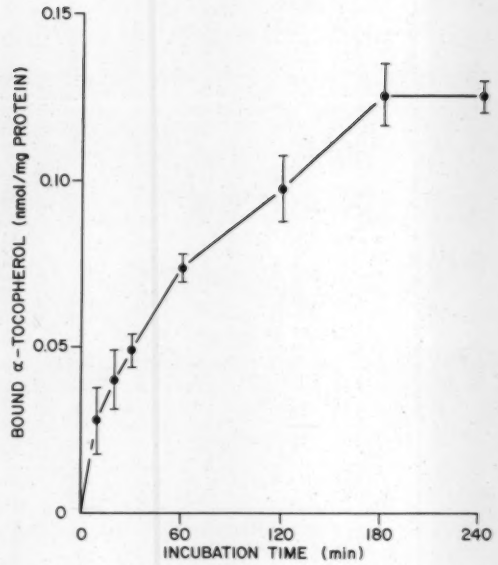


FIG. 2

Effect of different amounts of α-[^{3}H]-TBP in the transfer of α-tocopherol to liver microsomes. Microsomes were incubated at 37°C, for 180 min in 1 ml of a medium containing 100 µg of microsomal protein and different amounts of α-[^{3}H]-TBP in 0.1 M potassium phosphate buffer, pH 7.4. Each point represents the mean ± SD of three determinations.

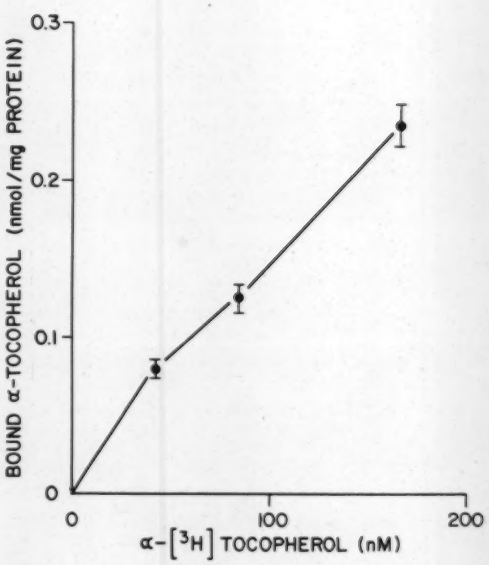


FIG. 3

Effect of different amounts of microsomes in the binding of α-tocopherol. Different amounts of microsomes were incubated at 37°C, for 180 min in 1 ml of a medium containing 83.5 nM of α-[^{3}H]-tocopherol as α-[^{3}H]-TBP in 0.1 M potassium phosphate buffer, pH 7.4. Each point represents the mean ± SD of 3 to 6 experiments.

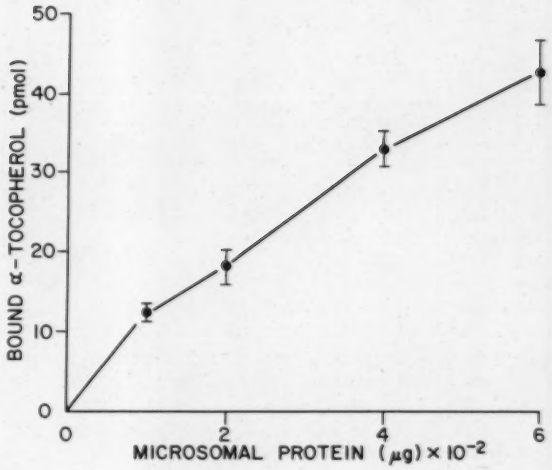


FIG. 4

Figure 4. Temperature-dependence of α-tocopherol transfer to liver microsomes. Microsomes were incubated at the indicated temperatures, for 180 min in 1 ml of a medium containing 100 μg of microsomal protein and 83.5 nM of α-[^{3}H]-tocopherol, as α-[^{3}H]-TBP in 0.1 M potassium phosphate buffer, pH 7.4. Each point represents the mean ± SD of 3 to 4 determinations.

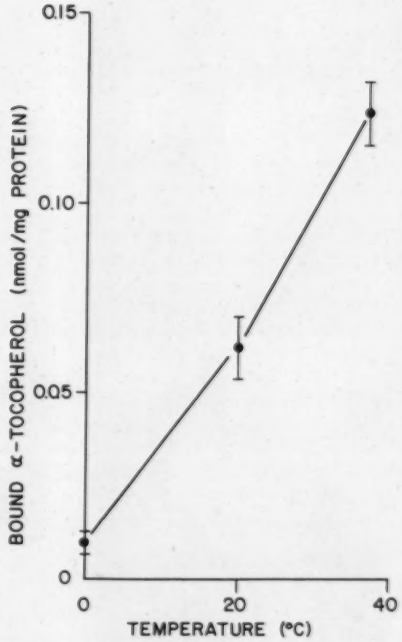
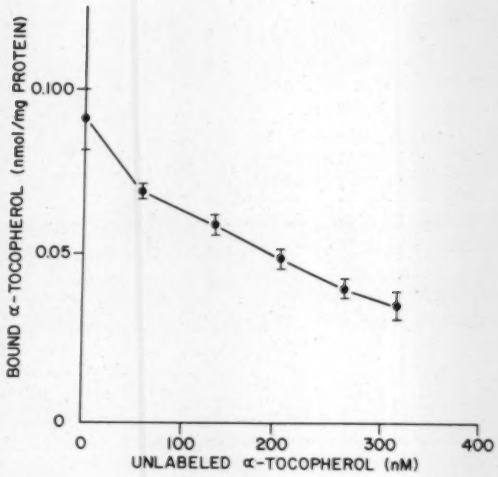


FIG. 5

Effect of unlabelled α-tocopherol on the transfer of α-[^{3}H]-tocopherol to liver microsomes. Microsomes were incubated at 37°C, for 180 min in 1 ml of a medium containing 100 µg of microsomal protein and 83.5 nM of α-[^{3}H]-tocopherol, as α-[^{3}H]-TBP, in the presence of different amounts of unlabelled α-tocopherol (added as α-TBP) in 0.1 M potassium phosphate buffer, pH 7.4. Each point represents the mean ± SD of 3 experiments with the exception of the initial value (6 experiments).



DISCUSSION

The results reported herein indicate that a purified preparation of α-[^{3}H]-TBP, labelled in vivo with α-[^{3}H]-Tocopherol, is able to transfer the vitamin to liver microsomes.

The nature of the bond between α-tocopherol and α-TBP is not known, but appears to be rather strong, as assessed by its persistance even after ammonium sulfate fractionation, as well, gel filtration, ion exchange chromatography, and extensive dialysis. Even though the incubation conditions are probably not the optimal ones the transfer of α-tocopherol to microsomes occurs. This could indicate that the affinity of low-vitamin E microsomes, or membranes in general, for the vitamin is even greater than that of α-TBP.

Under the conditions of incubation the amount of bound α-[^{3}H]-tocopherol was linearly proportional to the concentration of α-[^{3}H]-TBP and to the amount of microsomal protein. The transfer of vitamin was dependent on temperature of incubation, indicating that the mechanism of transfer is a specific process. A 3.8 fold increase of unlabelled α-tocopherol (bound to the same protein) was enough to produce a decrease of 62% in the binding of α-[^{3}H]-tocopherol to microsomes. Therefore the binding of α-tocopherol to microsomes is saturable. These findings are more consistent with the view that α-tocopherol is bound to microsomal proteins, as had been suggested by Timberg and Barber (13), rather than in agreement with mere solubility in, or non-especific intercalation in the membrane phospholipid bilayer of micromes as suggested by Murphy and Mavis (10).

Under our conditions of incubation 3 hours were needed to reach equilibrium. However, Murphy and Mavis (7) reported that only 4 min were necessary to reach equilibrium, with spontaneous transfer, in the absence of α-TBP, reaching the same level in 20 min. Mowri et al (6) reported that 20 min were needed for equilibration but spontaneous transfer activity was much lower than that reported by the previous authors. These discrepancies could well be due to different methods used to study the transfer activity of α-TBP. As well, both Murphy and Mavis (7) and Mowri et al. (6) used α-[^{3}H]-tocopherol bound to liposomes to study this transfer activity. This approach has been questioned by

Conrad and Singer (14) who have cast serious doubt on the use of liposomes as models for biomembranes.

The molecular weight of liver α-TBP was estimated by several workers (3-7) to be in the range of 30,000 to 34,000. It could be detected only in liver. However, Mowri et al (6) reported that cytosol preparations from liver, heart, spleen and lung were able to transfer α-tocopherol from labelled liposomes to liver mitochondria. A more precise determination of the molecular weight and other physico-chemical properties of this protein must await a pure preparation of α-TBP. Also, this could lead to the development of immunochemical methods for the assay of the protein and facilitate determination in other tissues.

The purification described here is an abbreviation of a more detailed method developed in this laboratory. This longer method has enabled us to produce only small amounts of a nearly homogenous protein fraction. By increasing the amount of starting material and modifying the protocol, we hope to overcome this problem.

The data presented herein, together with those of Mowri et al (6) and Murphy and Mavis (7) strongly suggest that the metabolic role of this protein is to act as an intracellular carrier of α-tocopherol. However, other possibilities, such as this protein playing a more specific metabolic role, or the existence of an enzyme with α-tocopherol as a prosthetic group, must also be considered.

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BLOOD PRESSURE DIFFERENCES IN SPONTANEOUSLY HYPERTENSIVE RATS PRODUCED BY VARYING THE SOURCE OF DIETARY FAT

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ABSTRACT

The effect of various dietary fats on blood pressure was studied in spontaneously hypertensive rats (SHR) and normotensive Wistar rats which were fed six different diets. Three diets contained liquid vegetable oil (5% olive oil, 5% corn oil or 17% corn oil) and three of the diets contained solid fat (5% butter, 5% hard margarine high in saturated fat or 5% soft margarine high in polyunsaturated fat). Diets were fed for 16 weeks beginning at 4 weeks of age. SHR fed 5% olive oil, 5% hard margarine or 5% butter in the diet had significantly lower blood pressures than those fed 5% or 17% corn oil or 5% soft margarine. The effects observed appear to be related to the amount of linoleic acid (LA) in the various dietary fats studied. SHR fed diets in which the LA content of the diet was low had the lowest blood pressures. Since LA is the dietary precursor of the prostaglandins, and one of the prostaglandins which has vasoconstrictor properties has reported to be elevated in SHR, increased precursor in the diet could result in increased biosynthesis of this prostaglandin. Increased vasoconstrictor activity would in turn aggravate the hypertension of SHR.

KEY WORDS: hypertension, fat, linoleic acid, prostaglandins

INTRODUCTION

The role of various dietary lipids in lowering blood cholesterol levels to reduce the risk of atherosclerosis has received a good deal of attention for a number of years. Little attention has been devoted, however, to determining the effects of various dietary fats on other physiological parameters involved in cardiovascular disease, such as hypertension. Recent experimental (1) and clinical (2) reports suggest that blood pressure can be influenced by the type of fat in the diet. Vegetable fats with a high content of linoleic acid (LA) have been reported to modify the blood pressure of rats with salt-induced hypertension and humans with mild essential hypertension. We have previously reported that a low LA diet fed to normotensive Wistar rats on a low sodium diet results in a significant increase in blood pressure within 7 to 10 weeks and this response is exaggerated by adding salt to the diet (3).

Commercial margarines contain varying amounts of LA depending on the type of vegetable oil used in their manufacture. Fats such as sunflower, corn and soybean oils contain relatively high amounts of LA whereas olive oil contains

mostly monunsaturated fats with only small amounts of LA. Palm and coconut oils which are not only used in margarines but also in many processed foods such as coffee whiteners, whipped toppings, bread, cakes, cookies, crackers, etc. are highly saturated and contain very little LA. Since dietary LA is the precursor for the synthesis of endogenous prostaglandins (PG) which have been implicated in kidney function and blood pressure regulation (4,5), it is of interest to investigate what effect, if any, these dietary fats have on blood pressure.

TABLE 1
Composition of the basic diet

Ingredient:	%
Casein (vitamin free)	20.0
Salt Mix[a]	4.0
Vitamin Mix[b]	2.5
Fat	see Table 2
White Sugar	to 100.0

[a]Bernhart & Tomarelli (8); [b]Box & Mogenson (9)

METHODS

Animal Care and Diets Experiments were conducted on 48 young male spontaneously hypertensive rats (SHR) of the Okamoto strain (6) from the Charles River Breeding Laboratories, Willmington, Mass. and 48 normotensive Wistar rats purchased from Canadian Breeding Laboratories, St. Constant, P.Q. Wistar rats were chosen as the controls instead of the usual Wistar-Kyoto (WKY) strain for several reasons. In a previous experiment, WKY rats had proven to be very susceptible to infection; in our current experiment the main objective was to make a comparison among various diets rather than between strains of rats. The animals were obtained shortly after weaning when they were approximately 4 weeks of age and weighed 110 + 2.5 g (SHR) and 125 + 5 g (Wistar). Upon arrival each of the two groups was divided into 6 groups (8 rats per group) of equal body weight. Although the SHR were slightly lighter in weight initially than the Wistar rats there were no significant differences by the time the rats were 8 weeks old (see Fig. 2). They were housed in individual stainless steel metabolism cages in a room illuminated for 12 hr per day and kept at 23 + 2°C. Fresh diet and water were available ad libitum. Tap water was supplied in glass bottles fitted with stainless steel drinking spouts. Experimental diets were fed on arrival and were supplied in glass jars fitted with spill-resistant lids. The composition of the basal diet which was low in sodium (0.08%) is shown in Table 1. The total fat content of each diet and the polyunsaturated fatty acid (PUFA) content which was considered to be LA, is shown in Table 2. Diets were prepared each week and stored at 5°C.

TABLE 2
Composition and level of fats used in diets

	Diet	Level in Diet (%)	PUFA[a] Content of Fat (%)
Liquid Vegetable Oils:			
Olive Oil	1	5	7.0
Corn Oil	2	5	57.0
Corn Oil	3	17	57.0
Solid Commercial Spreads:			
Hard Margarine[b]	4	5	2.8
Soft Margarine[c]	5	5	52.6
Butter (unsalted)	6	5	3.0

[a]PUFA analysis of oils by gas chromatography and of solid fats by the lipoxidase method (10).
[b]Composition according to label: palm oil 80%, water less than 16%, salt 3%, whey powder 1%, lecithin 0.2%.
[c]Composition according to label: liquid vegetable oil 70%, palm oil not more than 10%, water not more than 16%, salt 1.8%, whey powder 1.4%, lecithin 0.2%, potassium sorbate 0.1%, monoglycerides 0.1%.

Experimental Procedures Systolic blood pressures were measured every 2 weeks using a tail-cuff plethysmographic apparatus. Body weights were measured once per week. 24-hr food and water intakes were measured for 2 consecutive days on alternate weeks and the daily measurements averaged for each rat. 24-hr urine samples were collected at three different periods during the experiment (at the 2nd, 8th, and 14th experimental week). Urine was analyzed for sodium and potassium using a flame photometer. The amount of these electrolytes excreted was expressed as a percentage based on the amount of sodium and potassium consumed in the food for each rat. The kidneys, heart, liver and spleen were removed from the animals at the conclusion of the experiment and weighed. All

quantitative results were expressed as means + SEM for each group and were analyzed using Duncan's multiple range of analysis of variance (7).

RESULTS

The systolic blood pressures of rats fed the liquid vegetable oil diets are shown in Fig. 1A and those of the rats fed the solid fat diets are shown in Fig. 1B. The blood pressures of all SHR rose continually during the first 6 wk of the dietary regimes and plateaued at a significantly higher level than those of the Wistar rats. SHR fed Diets 1, 4, and 6 (olive oil, hard margarine and butter), which provided a low level of PUFA, had the lowest blood pressures whereas those fed Diets 2, 3, and 5 (5 and 17% corn oil and 5% soft margarine) which provided higher levels of PUFA had higher blood pressures.

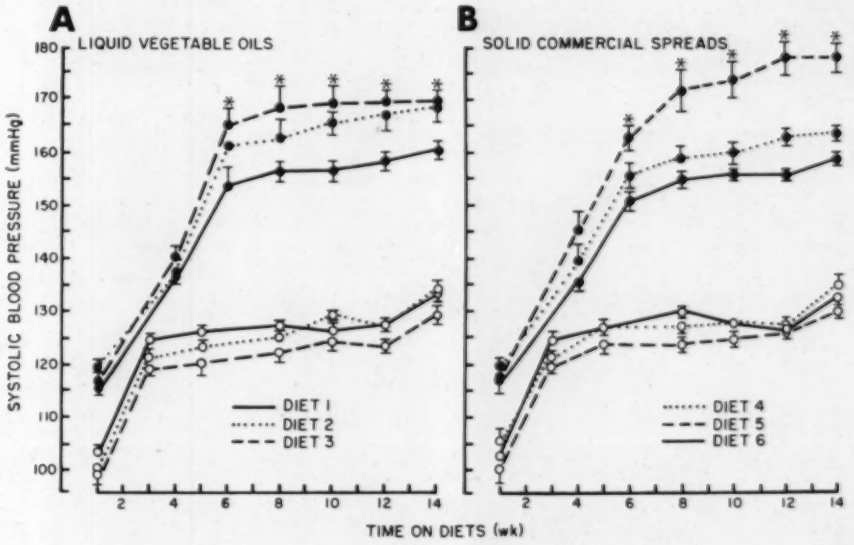

FIG. 1

Systolic blood pressures of SHR (solid circles) and normotensive Wistar rats (open circles) fed diets containing liquid vegetable oils (A) or solid commercial spreads (B) as the fat source for 14 wk. There were 8 rats in each dietary group. Vertical lines represent SEM. *indicates $P<0.05$ as compared with SHR fed the 5% olive oil diet in A or the 5% butter and hard margarine diets in B during the same time period. Diet 1 = 5% olive oil; Diet 2 = 5% corn oil; Diet 3 = 17% corn oil; Diet 4 = 5% hard margarine; Diet 5 = 5% soft margarine; Diet 6 = 5% butter (see Table 2 for LA content of fats).

The blood pressures of the normotensive Wistar rats plateaued after the first 3 wk on the dietary regime and thereafter the pressures remained stable with relatively small differences among dietary groups, although the highest blood pressure readings tended to be in the groups fed diets with the lowest LA content (olive oil and butter) as we observed previously (3).

The Wistar rats weighed slightly more than the SHR initially but after 4

weeks on the experimental diets there were no significant differences (Fig. 2). Thereafter, the SHR tended to weigh less than the Wistar rats showing that the hypertension of the SHR was not due to their being more obese than their normotensive counterparts.

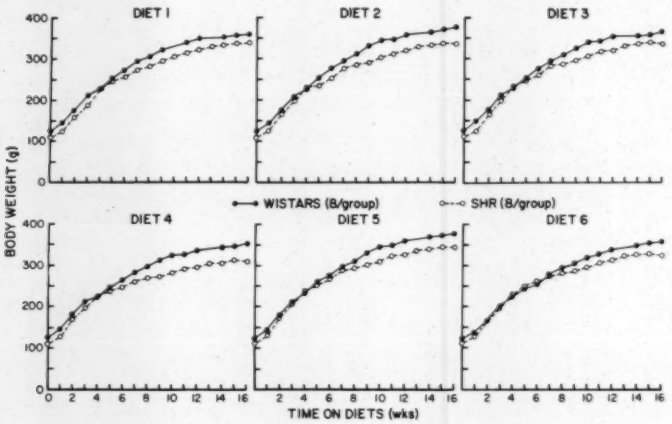

FIG 2

Body weights of SHR (open circles) and normotensive Wistar rats (solid circles) during the experiment. There were no differences between SHR and Wistar rats in the 6 different diet groups for the first 6 weeks of the experiment. See text for other comments and Tables 1 and 2 for composition of diets.

As shown in Fig. 3 Wistar rats tended to drink more water and excrete more urine than SHR on all diets but due to wide individual variations within groups these differences usually did not attain statistical significance.

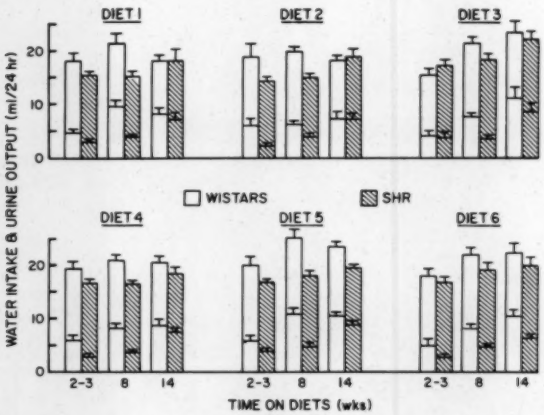

FIG. 3

Water intakes (ml/rat/24 hr) as shown by the full length columns and urine volumes as shown by the inner columns of SHR (hatched) and Wistar rats (open) measured at three periods during the experiment. Vertical bars represent SEM.

TABLE 3

Food intake (g/rat/24 hr) of Wistar and SHR rats fed diets varying in the source of dietary fat

Time on Diets		Liquid Vegetable Oils			Solid Commercial Spreads		
		Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
2-3 wk	Wistar	12.8 ±0.62	11.9 ±0.83	10.3 ±0.49	12.8 ±0.37	12.6 ±0.75	12.9 ±0.74
	SHR	16.3* ±0.49	15.4* ±0.33	15.0* ±0.27	15.4* ±0.38	15.3* ±0.62	15.5* ±0.42
8 wk	Wistar	14.8 ±0.69	15.4 ±0.32	15.0 ±1.89	16.9 ±0.48	16.4 ±1.19	16.3 ±0.45
	SHR	13.5 ±0.57	12.5 ±0.50	12.3 ±0.50	12.6 ±0.50	12.3 ±0.40	13.5 ±0.50
14 wk	Wistar	14.4 ±0.30	14.4 ±0.32	14.4 ±0.30	15.5 ±0.50	15.6 ±0.38	15.5 ±0.33
	SHR	13.4 ±0.63	14.0 ±0.53	13.3 ±0.53	14.8 ±0.37	14.6 ±0.32	14.0 ±0.19

Results expressed as mean ± SEM. See tables 1 and 2 for description of diets. *$P<0.05$ as compared with similar value for Wistar rats.

Although SHR ate more than Wistar rats during the first 2-3 weeks of the experiment (Table 3) they excreted significantly less sodium and potassium (Fig. 4). This was the period when blood pressure was rising rapidly (see Fig. 1). After two months on the various diets, SHR increased their excretion of both electrolytes but the percent excreted was still less than that of the Wistar rats.

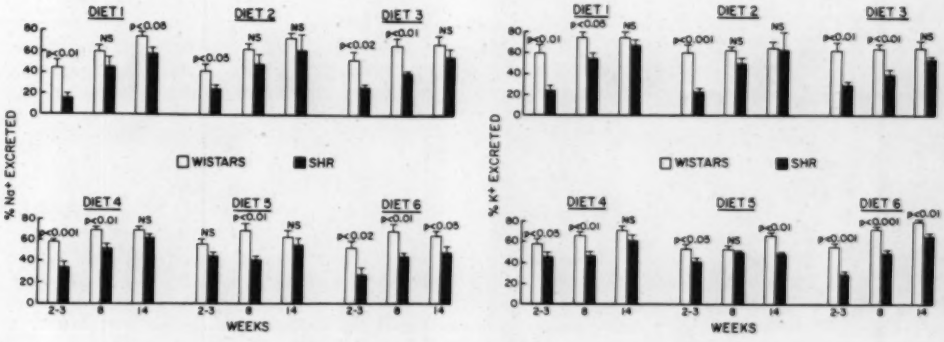

FIG. 4

Percent sodium (left) and potassium (right) excreted by SHR (solid columns) and normotensive Wistar rats (open columns) fed six different diets which varied in the source of fat. Urinary Na+ and K+ was measured at three different periods during the experiment. Vertical bars represent SEM. Values above columns indicate statistical difference between SHR and Wistar rats fed the same diets. NS indicates no significant difference.

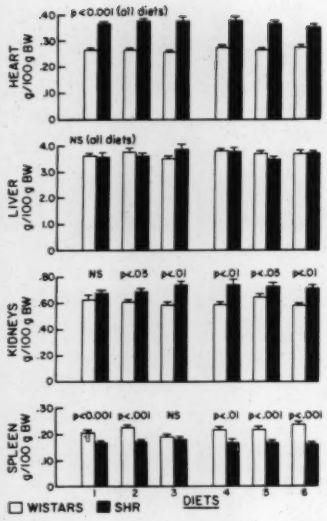

FIG. 5

Weights of heart, liver, kidneys, and spleen expressed as g/100 g of body weight. Open columns represent organ weights of Wistar rats and solid columns those of SHR. Vertical lines represent SEM. Values above columns indicate statistical difference between SHR and Wistar rats fed the same diet. NS indicates no significant difference.

The kidneys and hearts were heavier and the spleens lighter in weight in the SHR as compared with the Wistar rats when these organs were removed at the conclusion of the experiment (Fig. 5).

DISCUSSION

The results of the present study demonstrate that systolic blood pressure of SHR varies according to the dietary source of fat. SHR fed diets containing olive oil or butter, which have a relatively low LA content, developed a lesser degree of hypertension than SHR fed dietary fats with a higher LA content. This is consistent with our earlier observations in which we showed that SHR fed an 8% coconut oil diet developed hypertension to a lesser degree than those fed a 17% corn oil diet (11). In addition these experiments are the first to report differential effects on blood pressure of SHR fed commercial spreads. In this respect it is interesting to note that the highest blood pressures were obtained in SHR fed 5% soft margarine in the diet, even though it did not provide as high a level of LA in the diet as did the 17% corn oil. This suggests the possibility that other components of commercial spreads with a high LA content may also contribute to the elevated blood pressure.

It should be pointed out that the effects of various fats on blood pressure of SHR are the opposite to those observed during salt-induced hypertension (1, 3). One possible explanation of this seeming paradox could be that there is a difference in prostaglandin (PG) synthesis in these two models of hypertension. In the kidney, PGs appear to modulate blood pressure by a balance between vasodilatory and vasoconstrictive PGs (12). PGE_2 is a vasodilator, increasing renal blood flow and participating in the regulation of water and salt excretion, while PGF_{2a} is a vasoconstrictor (13). In normotensive rats there is a predominance of PGE_2 synthesized in the kidney as compared with PGF_{2a} whereas in SHR there is an increased synthesis of PGF_{2a} and thus a decreased PGE_2/PGF_{2a} ratio (14). A number of investigators have shown that the amount of LA in the diet influences the amount of PGs synthesized in the body, due to the fact that LA is the dietary precursor of the PGs (15, 16). Thus an increase in dietary LA may aggravate vasoconstriction and arterial hypertension

in SHR by decreasing the PGE_2/PGE_{2a} ratio whereas a similar increase in dietary LA may increase the PGE_2/PGF_{2a} ratio during salt-induced hypertension. The recent work of Schoene and colleagues suggests that this may indeed be the case (17).

Although significant differences in blood pressure of SHR were observed among the various dietary groups, other parameters were similar. An exception was the excretion of sodium and potassium, although the differences were not consistent over the various periods when they were measured. Excretion of both of these electrolytes increased with time on all of the diets but were still lower than the control Wistar rats. Dietz and coworkers have made similar observations on sodium excretion and retention in SHR (18). They found that young SHR excreted lower amounts of sodium during the early phase of hypertension than during the later stages and suggest that young SHR can excrete sodium only if the blood pressure is sufficiently elevated. This implies that SHR have a primary defect in the renal handling of sodium which may be a factor in the initial phase of hypertension. The hypertrophy of hearts and kidneys of SHR which we observed has also been reported by Ueda (19, 20) who noted that heart weight increased in proportion to blood pressure but kidney weight varied according to the composition of the diet. There was an indication in our experiments that the amount of kidney hypertrophy increased with the amount of LA in the diet.

In conclusion these experiments show that blood pressure in SHR can be altered by varying the source of dietary lipids whether they are in the form of vegetable oils or solid fats. Diets containing a high level of LA in the fat aggravate the hypertension while a low level of LA ameliorates the hypertension. Although the mechanism of these effects is not completely understood, it may be related to an abnormality in PG synthesis which has been observed in SHR. Since a defect in renal PG metabolism may be a factor in the development of high blood pressure in human essential hypertension (21) as well as in the New Zealand model of genetically hypertensive rat (22), blood pressure in other forms of hypertension may also be affected by the source of dietary lipids. However, further research is necessary to elucidate the mechanisms by which LA exerts its physiological effects on blood pressure in all models of hypertension.

ACKNOWLEDGEMENTS

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CONFERENCE SUMMARY

International Food Allergy Symposium IV

The symposium was organized by the American College of Allergists and was held July 25-29, 1982 in picturesque Vancouver. In the opening session, R.K. CHANDRA described the process of macromolecular absorption and the various factors which determine the development of allergic disease. He emphasized the critical importance of early feeding practices and presented data showing the reduction in incidence both of allergy and infection in exclusively breast-fed infants. JOHN GERRARD outlined the various ways in which allergic disorders may present, the manifestations can vary from acute anaphylaxis to vague multi-system symptoms. JOSEPH SIDOROV described beneficial effects of sodium cromoglycate in patients with ulcerative colitis receiving corticosteroids. The criteria for selection of patients who would benefit from such combined therapy are not established. C. COLLINS-WILLIAMS suggested that oral sodium cromoglycate may be employed as a diagnostic tool in patients suspected to have food allergy.

ROBERT HAMBURGER emphasized that the current management of food allergy depends on a careful history, diet diary, elimination diets, antihistaminics and oral cromolyn. Atopic food allergy is often associated with elevated total serum IgE but low levels with IgE directed against one allergen may be observed, the total and specific IgE levels being independently regulated on a genetic basis. DEAN METCALFE described the distribution of mast cells in the gastrointestinal tract. These cells degranulate in food hypersensitivity, the process being mediated by IgE antibodies, immune complexes and the food antigen itself. The consequent hypersensitivity response increases permeability to macromolecules. Measures to prevent reactions to foods would include elimination of offending foods, drugs such as cromolyn and aminophylline that prevent mast cell degranulation, those that prevent the production of secondary mediators such as prostaglandins, e.g. aspirin, and finally drugs that block the action of released mediators, e.g. antihistamines.

JONATHAN BROSTOFF demonstrated the frequent presence of IgE immune complexes in patients with a variety of atopic diseases. The complexes were poorly cleared. IgE complexes were found in a proportion of patients with migraine. DEREK WRAITH showed that in patients with eczema due to food allergy most of the specific food IgE antibodies were elevated. Allergy to foods may underlie joint disease in some individuals. OSCAR FRICK described non-IgE mediated food allergy. Raji cell assay for immune complexes was positive in a majority of patients with delayed onset food hypersensitivity symptoms. Sera of these patients also had significantly greater binding of radiolabelled anti-beta lactoglobulin which increased after cow's milk ingestion. JAMES BRENEMAN described his experience with patch tests using individual foods suspended in dimethyl sulfoxide and showed that this procedure may provide a valuable screening method for evaluating suspected allergy to foods. Immunological studies seemed to verify the accuracy and reliability of the patch tests.

AUBREY KATZ discussed the clinical features, pathogenesis, diagnosis and management of gluten-sensitive enteropathy. Both malabsorption and intestinal villus atrophy improve on exclusion of foods containing gluten, rye, oats and barley. He emphasized that there are many other causes of a flat villus pattern.

Genetic predisposition is suggested by the linkage of this syndrome with histocompatibility antigen HLA-B8,DW3. THEODORE BAYLESS talked about lactose maldigestion and milk intolerance as causes of gastrointestinal symptoms, including abdominal bloating, gaseousness and cramps. Diarrhoea may be observed and milk ingestion may be employed as a cathartic. The diagnosis can be established by lactose tolerance test, breath hydrogen analysis or in vitro assays of small intestinal mucosa showing low levels of lactase.

In the final session, ROBERT OWEN showed captivating ultrastructure of the intestinal mucosa, lymphocytes and macrophages and how a pathogen such as Giardia lamblia evades these protective barriers to establish camp and cause symptoms. He described the mechanisms of antigen penetration which is a prerequisite to the occurrence of sensitization and inflammatory response. RANJIT CHANDRA outlined various host defense mechanisms in the gastrointestinal tract including gastric acid and proteolysis, intestinal microflora, bile acids, mucus, ciliary activity, secretory IgA antibodies, T lymphocytes, macrophages and other cells. Hepatic network provided a second barrier. These defenses are immature early in life. During this critical period of suboptimal protection, colostrum and breast milk shade the infant from infectious agents and food antigens. He described various inherited and environmental factors which interfere with intestinal defense and the consequences of impaired mucosal immunity.

The lectures given at the International Food Allergy Symposium IV are available on tapes. Direct inquiries to the American College of Allergists, 800 East Northwest Highway, Suite 101, Mount Prospect, Illinois 60056.

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COUNTRY REPORT

Egypt and Middle East

Serum glycoproteins were determined in 38 children with protein-calorie malnutrition (PCM) and in 58 normal control children. In PCM serum IgG and A were significantly decreased while serum IgM showed no significant change. The serum globulin fractions: alpha antitrypsin ceruloplasmin, alpha macroglobulin, and transferrin were all decreased in PCM, alpha acid glycoprotein was elevated. Haptoglobin was not affected. Their role in the modulation of lymphocyte responsiveness and their possible relation to the increased susceptibility of these patients to infection is discussed. [Domyatic, H., Heneidy, F., Khalafallah, Bahgat N. and Shenawy, R., Gaz. Egypt. Ped. Ass. 28:11, 1980.]

Renal function tests together with renal biopsy were performed on 45 infants and children suffering from PCM (Kwashiorkor, marasmus and marasmic kwashiorkor). Glomerular filtration rate was impaired in PCM, especially the kwashiorkor group. Renal tubular concentration was also impaired as evidenced by the constant low specific gravity of urine in all types of malnutrition. Blood serum uric acid concentration was reduced. Proteinuria and hypoalbuminemia were pronounced in PCM with edema. Renal biopsies revealed dilation and rigidity of glomerular capillaries, variability of glomerular size and thickening of the basement membrane. The tubules were dilated and their walls were thin and showed cloudy swelling with frequent vascular degeneration. Interstitial fibrosis was seen in a few cases from the kwashiorkor group only. This study revealed that significant morphologic changes occur in the kidney in severe PCM. Impaired renal function is most pronounced in kwashiorkor, confirming the relation between histopathologic changes and deranged renal function. [Behairy, F., Eissa, A., Eldiwani, K., Abdin, F., Abdel Hafiz, A., and Malky, F., Gaz. Egypt. Ped. Ass. 28:191, 1980]

A study of the effects of birth interval on the child's physical growth using various anthropometric measurements, was carried out on 838 infants and children, aged 0-24 months. Cases were divided at 3 months' intervals into 8 groups. Anthropometric measurements included body weight, length, head circumference, chest circumference, subscapular and triceps skin fold thickness and mid-upper arm circumference. As one moved from one age group to an older one, the gain in each body measurement was greater for infants following long birth interval in relation to their age matched cases following short birth interval. A statistically significant difference was present throughout nearly all of the second year of life indicating that children delivered following a long birth interval had better growth. Children with malnutrition, delayed teething, or rickets, had significantly shorter mean birth interval than others. [Behairy, F., El-Garhy, M., El-Mougy, M., El-Diwany, K., Khalifa, E., and Soliman, A., Gaz. Egypt. Ped. Ass. 28:233, 1980.]

In a group of kwashiorkor cases and normal controls, total phospholipid concentration as well as thin layer chromatography of phospholipid fractions in platelets and serum were performed and platelet functions were studied. Significant diminution of total phospholipids, sphingomyelin and phosphatidylcholine were found. Total phospholipids in platelet account for 11.2% that of serum. Sphingomyelin, phosphatidylcholine and ethanolamine were significantly diminished in platelets compared to serum values. The percent of platelet

phosphatidylcholine was lower than that in serum, while phosphatidyl ethanolamine showed a remarkable increase. There was a significant correlation between platelet aggregation and adhesiveness on the one hand and platelet phosphatidyl ethanolamine on the other hand in patients with purpura. [Khalil, M., Aref, M., Mahmoud, S., El-Sawy, M., Abdel Hay, M., Sidrak, M., Gaz. Egypt. Ped. Ass. 29: 35, 1981.]

Hepatic histological and histochemical examinations were carried out in twenty pellargic children before and after treatment. Necrotic foci, dilated blood sinusoids and irregular cords of hepatocytes were evident in all cases. There was fatty infiltration of the liver and a decrease in the amount of stored glycogen. Diminished activity of both alkaline phosphatase and succinic dehydrogenase were observed in the hepatocytes in contrast to the increased activity of alphaesterase and acid phosphatase. Follow up of the cases was carried out for 6-7 weeks after starting the treatment. Evaluation of the changes of liver biopsies after evident clinical recovery showed a considerable improvement in most of the pathological changes. [Aref, K., Soliman, S., Hamam, M., Shaban, M., Koraitim, Y., Kato, A., Hodeib, A. and Sherif, A. Gaz. Egypt. Ped. Ass. 29:103, 1981.]

A Saudi study dealt with the results of village surveys carried out in 3 different areas, a desert area, a mountainous area and a coastal area. 452 families were studied. Over 90% of the mothers were unable to read. Poor sanitary conditions were prevalent; piped water supply existed in 10% of the houses while 67% of the houses had no latrines. Extended families were the rule, often with three generations living together. The birth intervals seemed to be short and the mothers have an average of 5.5 children each. The age pyramid from the community revealed a wide base with 25% of the population less than 6 years of age indicating a high fertility rate and high wastage of child life. Infant mortality rates varied between 117 and 144/1000 live births. Below 5 mortality rates varied from 6.6 to 14.7/1000 population. Perinatal mortality rates which are likely to be under-estimated ranged between 41 and 61/ 1000 live births. Breast feeding for more than a year was the rule, 53 to 76% of all children were still breast-fed at 12 months of age. There was, however, a considerable interest in bottle feeding. Health facilities were frequently underutilized; 84% of births occur at home; vaccination coverage varied between 0.4 to 4.0%. Anthropometric evaluation of 827 preschool children revealed a point prevalence of acute malnutrition of 6%. The point prevalence of children with chronic malnutrition was 24%. Clinical examination of 256 preschool children revealed 3 children with clinical marasmus, 7 with general wasting, 2 with rickets and 2 children with goiter. There were no children with signs of vitamin A deficiency or clinical kwashiorkor. [Selenrus, F., Fougerouse, D., Saudi Med. J. 2:10, 1981.]

A small survey was instituted among mothers attending Riyad El-Kharj Hospital. Seventy-two percent of babies were found to be entirely breast-fed at the end of the first week. By 3 months only 42% were entirely breast-fed and this decreased further so that at one year 11% of babies were still receiving breast milk as the only source of milk. Few infants (12%) were entirely bottle fed at one week, but by six months 41% were fed in this way and by the age of one year 55% of babies had ceased to receive any breast milk. Only 50% of mothers gave a correct answer when asked how to prepare a milk powder bottle feed. The age at which solid food was introduced varied between 5 and 7 months. The majority of the mothers gave rice, bread or vegetable soup as the first foods. The survey indicated that in the urban hospital population studied, breast feeding was on the decline. [Lawson, M., Saudi Med. J. 2:26, 1981.]

A report from Egypt deals with the results of a follow up nutrition status survey of preschool children carried out in the summer of 1980 in two areas, one in Lower Egypt and one in Upper Egypt, originally surveyed during the winter of 1978. Both surveys were conducted by the Nutrition Institute of Cairo, with the Technical Assistance of the Nutrition Division, Center for Disease Control, Atlanta, in cooperation with the U.S. Agency for International Development. More than 1700 children were examined in each survey. The report presents information regarding prevalence and distribution of acute and chronic protein energy undernutrition and anemia in children 6 through 71 months of age during 2 periods 18 months apart as well as its interaction with diarrheal disease which is more prevalent in summer. Anthropometric data were analysed for 1783 children for the 1980 survey versus 1772 for the 1978 survey. Results indicated an increase in the mediun height for age of preschool children during the 18 month period, reflecting a small improvement in the long term nutrition status. Figures for severe chronic undernutrition (less than 85% of the NCHS/CDC reference median) which varied between 3% in Lower Egypt and 9% in Upper Egypt during the 1978 survey decreased to 2% and 6% respectively in the 1980 survey. On the other hand, there was an increased prevalence of acute wasting with a lower weight for age. While 6% in Lower Egypt and 15% in Upper Egypt had a weight for age below 75% in 1978, the figures were 11% and 19% respectively in the 1980 survey. Various factors might be involved; economic, social as well as disease prevalence, particularly diarrheal disease. The prevalence of recent diarrheal disease was higher in the 1980 survey and children with such a history were thinner and more likely to be wasted than children without diarrhea. Anemia both in mothers and children remained substantial in the 1980 survey and at a comparable level to 1978 survey findings. The mean hemoglobin level for preschool children varied between 10.8 to 11.2g%. More than 15% of mothers had a hemoglobin level below 11g%. [Report, Nutrition Institute, Ministry of Health, Egypt, March, 1981.]

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ANNOUNCEMENTS

Course on Practical Nutrition

A course entitled Practical Nutrition will be given in Lake Buena Vista, Florida, March 19-21, 1983. The faculty include Drs. G. Harvey Anderson, Bruce Bistrian, Ranjit Chandra, David Kritchevsky, Emanuel Lebenthal, Hamish Munro, Ananda Prasad, and Noel Solomons. The topics include Nutrient requirements and recommended dietary allowances, Nutrition of the elderly, Enteral and parenteral nutrition, Carbohydrate intolerance, Nutrition - immunity-infection interactions, Controversies in infant feeding, Nutritional anemias, Nutrition and gastrointestinal tract, Trace element in clinical practice, Nutrition and cancer, Obesity and others.

For further information and registration, contact Dr. R.K. Chandra, Janeway Child Health Centre, St. John's, Newfoundland, Canada A1A 1R8, Phone (709) 778-4519/4520.

Western Hemisphere Nutrition Congress

The next Congress will take place at Miami Beach, Florida, August 7-11, 1983. For further information, contact Dr. Philip White, Foods and Nutrition Department, American Medical Association, North Dearborn Street, Chicago, Illinois 60061.

Addendum: Article by S. Dutta et al.

The article entitled "Abnormal Dark Adaptation in Adult Patients With Protein-Energy Malnutrition: Corrected by Protein-Energy Repletion' authored by S.K. Dutta, R.M. Russell and V. Lakhanpal which appeared in Nutrition Research, 1:443-448, 1981, was supported by NIH grants No. 7R22 AM31081-01 as well as other funding sources.

Addendum: Article by J.S. Harrington and T. Nakamoto

The address of the corresponding author of the article entitled 'Effect of Liquid Protein Diet During Pregnancy on Newborn Rats' published in Nutrition Research, 2:163-173, 1982 is Dr. Tetsuo Nakamoto, Department of Physiology, Louisiana State University Medical Centre, 1100 Florida Avenue, New Orleans, Louisiana 70119.

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EDITORIAL COMMENTARY

Maternal Hematological Values and Pregnancy Outcome

In this issue of the journal is a carefully constructed study of the relationship of maternal hemoglobin changes and their relationship to infant birth weight. Although Higgins' study was retrospective in nature, it has several advantages over other attempts to interrelate pregnancy outcome and hematologic values observed during gestation. In this group of women, two values were obtained during pregnancy and thus, it was possible for the authors to link early and late pregnancy studies and make a direct comparison to pregnancy outcome with respect to birth weight. This inability to link values in the same individual and assess their role in pregnancy outcome has been a major fault in some large studies attempting, for example, to assess the efficacy of dietary supplements during pregnancy.

Another advantage of this study is the relative uniformity of antenatal care, particularly with respect to overall nutrition status. These women were followed in the same clinic, were registered early in pregnancy and were delivered at the same institution. It is of importance that all of these women were also enrolled in a dietary assessment program and to the degree it was possible, the various nutrition factors that could have a bearing on this study were controlled. It is possible, therefore, to deal more directly with the few variables of hemoglobin measurements, maternal weight gain and pregnancy outcome without undue reservations concerning the possible effect of other uncontrolled nutritional factors.

The study does point out, however, the need to take into account some analytical problems in such a study. These authors noted an increase in hemoglobin values among those women in the lowest category and a similar change toward mean values by a decrease in the group initially having the highest measured values. While they might attribute the "improvements" among the group having the lowest values to a favorable response to iron supplementation, they have no explanation for the decreasing high values. There is a well recognized analytical problem in making this specific kind of observation among any set of determinations there are bound to be some technical variation unrelated to true values. With a second determination it is likely that outlying inaccurate determinations will likely be more nearly the real value and thus near the average values for the whole population. When an investigator looks at the two extremes, therefore, and sees what happens to a subsequent value, it is likely that some subjects will be found to have moved toward the center and the whole population exhibits what is known as regression to the mean. While this observation does not negate the interpretation of this study, the behavior of individuals on the two extremes must be interpreted with caution.

These authors have provided a useful study in which a relationship between hemoglobin values, weight gain and pregnancy outcome can be assessed in a relatively well controlled situation. The authors have not gone beyond their data to draw further conclusions. Implicit in their data perhaps, might be the

conclusion that increased protein intake during pregnancy associated with plasma volume expansion and decreasing hemoglobin values produced the beneficial result of increased infant birth weight. Without a functional assessment of the consequences of this increased birth weight with respect to infant health this hypothesis, of course, remains untested.

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EDITORIAL

Iron Deficiency and Secretory Immunity

Few would dispute the contention that malnutrition alters immunologic reactivity and infectious disease resistance in the human host. Unfortunately, the complexity of human malnutrition, together with uncontrolled environmental factors (e.g. infection), confounds the interpretation of results of human studies, and forms a barrier to our elucidation of this relationship. Understanding of the impact of single nutrient deficiencies on immunity, therefore, has grown in direct proportion to the development of relevant animal models in which nutrition-immune response interactions can be studied. The most useful models have been devised to approximate an important human condition with respect to the importance of the nutrient studied, the chronicity and severity of the deficiency and the relevance of the immune responses measured for host anti-microbial defense.

A paper by Kochanowski and Sherman in this issue exemplifies this experimental approach. These authors report the effects of moderate maternal iron deficiency in rat dams on secretory immune substances in milk. The paper is important for three reasons: (a) the nutrient deficiency selected (i.e. iron) is one of the most common in both developed and developing countries; (b) the moderate nature of the deficiency mimics the most common form of iron deficiency seen in humans; and (c) the impact of maternal malnutrition on the adequacy of milk protective substances has not been determined.

Several previous studies have documented measurable impairment of secretory immunity in moderately and severely malnourished children and animals. Concentrations of secretory IgA, antibodies specific for certain respiratory viruses, and enzymes such as lysozyme were significantly reduced in the various secretions (tears, saliva, nasopharyngeal washes) studied (1-3). In the few published studies which have examined the impact of maternal malnutrition on secretory immunity in milk, the results have ranged from no effect (4) to significant alteration of both the quality and quantity of milk protective substances (5). Given the critical role of passive immunity during lactation in preventing both gastrointestinal and respiratory morbidity in suckling infants world-wide, it is imperative that we understand the influence of maternal nutritional status on milk immunity.

A review of the recent literature supports the generalization that iron deficiency is accompanied by significant impairment of humoral and cell-mediated immune responses (6). The close association between anemia and protein-calorie malnutrition in many populations suggests that iron deficiency may contribute to alterations of immune potential in malnourished humans. The relationship between iron nutriture and anti-microbial resistance is not a simple one, however, since factors such as the availability and saturation of iron-binding proteins, the iron requirements of the microbe, and the comparative affinities of host and microbial iron chelators may be more important than iron status *per se* (7).

The paper by Kochanowski and Sherman represents one of the first attempts to determine the effect of dietary iron deficiency on secretory immunity. While their deficient diet resulted in significant reductions in milk iron, serum and

liver iron and hemoglobin in the suckling pups, it was not severe enough to impair growth during lactation. The authors found that moderate iron deficiency did not alter appreciably immunoglobulin and enzyme levels in either the serum, milk, or saliva of deficient dams. Most significantly, IgA levels in milk and saliva were identical in normal and iron-deficient dams. These important observations certainly deserve to be pursued in this and other models. An earlier study (8) had shown reduced nasopharyngeal secretory IgA-antibody levels following immunization with live attenuated measles vaccine in a group of iron-deficient subjects. Among the critical experimental questions which should be examined are the effects of iron deficiency on secretory volume, the response of specific secretory IgA to antigenic stimulation in iron-deficient animals, and the response of iron-deficient dams and pups to infectious challenge of the respiratory and/or gastrointestinal tracts with microbes representing a range of iron requirements.

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MATERNAL HAEMOGLOBIN CHANGES AND THEIR RELATIONSHIP TO INFANT BIRTH WEIGHT IN MOTHERS RECEIVING A PROGRAM OF NUTRITIONAL ASSESSMENT AND REHABILITATION

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ABSTRACT

The haematological changes seen during pregnancy were studied in 1112 women registering in the public clinic of a large urban teaching hospital during the first or second trimester. In addition to routine antenatal care the women received nutritional services from the Montreal Diet Dispensary Program. Maternal haemoglobin level was affected by both iron deficiency and haemodilution, attributable to plasma volume expansion; diuretics and salt restriction were not used. There was no relationship between initial haemoglobin and infant birth weight. However, this was probably due to the nutritional therapy (protein, energy, iron and folic acid) that these women received during pregnancy. Birth weight was inversely related to late antepartum haemoglobin ($p < 0.01$) and to change in haemoglogin ($p < 0.025$). The women who had the greatest total increase in protein intake during pregnancy had the greatest fall in haemoglobin, presumably attributable to plasma volume expansion. In the present population there was no relationship between iron deficiency, as evidenced by decreased mean corpuscular volume or haemoglobin content, and pregnancy outcome.

KEY WORDS: birth weight, dietary protein, haemoglobin, iron, pre-natal care

The haemoglobin level is a concentration reflecting the relationship between plasma volume and total red cell mass (1,2). During pregnancy, both plasma volume (3) and red cell mass increase (1). The changes in plasma volume occur earlier

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and are usually of a greater magnitude with the result that maternal haemoglobin falls during pregnancy, reaching its lowest level at the start of the third trimester (1,2). Red cell mass and its haemoglobin content are affected by nutrient intake, including iron, folic acid and vitamin B_{12} (1,2,4). Plasma volume is affected by the use of diuretics (5), as well as by nutrient intake (6).

Whilst examining the effects of a program of maternal nutritional rehabilitation on the outcome of pregnancy, we observed an inverse relationship between late antepartum haemoglobin and the birth weight of the infant. These mothers were carefully monitored from the dietary standpoint, received multiple vitamin, iron and mineral supplements and were not salt restricted or given diuretics. We therefore considered that this population was particularly suitable for an examination of haematological changes and their relationship to infant birth weight and pregnancy outcome.

SUBJECTS AND METHODS

All mothers were patients of the public obstetrical clinic of the Royal Victoria Hospital, Montreal, between 1963 and 1975 inclusive. All registered during the first or second trimester of pregnancy and were delivered at the Royal Victoria Hospital. The women were also enrolled in a program of maternal nutritional assessment and rehabilitation run by the Montreal Diet Dispensary. Details of this program have been published elsewhere (7,8).

All women were given a prenatal vitamin and mineral supplement (Latan, Ayerst Laboratories, Saint-Laurent, Quebec) to be taken daily. The supplement contained vitamins A, B_{12}, C, D, thiamine, riboflavin, niacinamide, pyridoxine, folic acid 2 mg, plus 65.75 mg iron (as ferrous fumarate) and 250 mg calcium (as calcium carbonate). The women were seen by the dietician at each antenatal visit and encouraged to take the supplement, in addition to the specific dietary advice offered. Nutrient intakes were assessed at each visit (9,8), each woman making an average of six visits.

The haematological measurements were part of the routine antenatal care and the results were abstracted from the mothers' hospital records. An initial haemoglobin was available for most cases. However, in some women, a late antepartum (>33 (mean 36) weeks gestation) haemoglobin was not recorded. This paper will report on the 1112 cases where both haemoglobin values were recorded. All the haematological parameters were measured in the Royal Victoria Hospital Haematology Laboratory using standard techniques (9). Haemoglobin and haematocrit were measured on all specimens and red cell count on many of the blood samples. The haematocrit data is not presented in this paper, since it parallels the haemoglobin data and adds nothing further. The red cell counts were used to calculate the red cell indices, mean corpuscular volume (MCV) and mean corpuscular haemoglobin content (MCH).

This study was restricted to women giving birth to live singeltons in order to avoid the potentially confounding effects of multiple births on the birth weight data. All the women were healthy. Specifically, none were suffering from heart disease, pulmonary disease, renal disease or hypertension, factors that might affect haemoglobin or infant birth weight.

Statistical analyses were performed using one-way analysis of variance, co-variance (10) and step wise regression.

RESULTS

No significant relationship between initial haemoglobin and infant birth weight was seen (Table 1). Over 13% (144) of the women had initial haemoglobin <11 g/dl, and in terms of red cell indices, approximately 40% of these women had evidence of iron deficiency anaemia (MCV<80.8 fl or MCH<26.4 pg). The normal values used were for MCV 90.4 gl (range 80.8-100) and MCH 30.2 pg (range 26.4-34.0) (9). The women in the lower 3 haemoglobin categories (initial haemoglobin <12 g/dl) showed a mean increase in haemoglobin during pregnancy and the 28 women in the lowest haemoglobin category showed an improvement in their mean red cell indices to within normal range. The women with the lowest haemoglobin had lower pregravid weight ($p<0.001$).

A relationship was found between the change in maternal haemoglobin concentration and infant birth weight ($p<0.025$) (Table 2); the greater the decrease in haemoglobin, the higher the mean infant birth weight. Analysis of co-variance controlling for maternal pregravid weight and weight gain did not alter this inverse relationship between infant birth weight and the change in maternal haemoglobin concentration. Similarly, a significant inverse relationship was seen between late antenatal haemoglobin and the infant's birth weight ($p<0.01$) (Table 3). No differences were seen in low birth weight rates.

Mean daily protein intake was 100±20 g and energy 11.6±2.1 MJ. There were no significant differences in total protein or energy intakes over the entire pregnancy between the 6 different haemoglobin change groups. However, there was a relationship between the total increase in protein ($p<0.01$) (but not energy, $p=0.07$) intakes during the Montreal Diet Dispensary service and change in haemoglobin (Table 2). Greater increases in protein were associated with a greater fall in haemoglobin. Step wise linear regression was also carried out to examine the relationship between birth weight, the maternal haemoglobin variables and dietary intake. Birth weight was predicted by the following parameters, in order of significance; change in haemoglobin ($p<0.001$); and total increase in protein ($p<0.01$). The next 2 parameters identified were antepartum Hb and total increase in energy. However, neither were statistically significant. Initial haemoglobin did not predict infant birth weight.

DISCUSSION

Maternal haemoglobin concentration during pregnancy reflects the relative proportions of total red cell mass and plasma volume (1). Both have been shown to increase in normal pregnancy (1,3). Since the increase in plasma volume precedes and is usually proportionately greater than the increase in red cell mass, maternal haemoglobin levels tend to fall during pregnancy (1,2). It has been suggested that women with large plasma volumes produce large babies (11,12). Furthermore, women with inadequate haemodilution tend to have foetally growth-retarded babies (12,13,15,16). Protein restriction and total food restriction have been shown to reduce plasma volume and conceptus weight in pregnant rates (6). A maternal antepartum haemoglobin >13 g/dl has been reported as being associated with an adverse pregnancy outcome (15,16). The present data (Table 3) would support these findings, at least in terms of mean infant birth weight. This high level of antenatal haemoglobin could be due either to

TABLE 1

The relationship between initial haemoglobin and infant birth weight

Parameter	Initial Haemoglobin (g/dl)						
	<10.0	10.0-10.9	11.0-11.9	12.0-12.9	13.0-13.9	≥14	p[2]
Patient numbers	28	116	313	416	194	45	
Pregravid wt (kg)	57.2±12.0[1]	54.2±9.6	56.3±10.8	57.3±10.5	59.5±13.6	61.1±15.8	<0.001
Initial Hb (g/dl)	9.2± 0.7	10.6±0.3	11.5± 0.3	12.4± 0.3	13.4± 0.3	14.4± 0.5	<0.001
Antepartum Hb (g/dl)	10.7± 1.3	11.1±1.0	11.7± 0.9	11.9± 0.9	12.4± 1.0	12.8± 1.1	<0.001
Birth wt (g)	3278±373	3309±478	3371±499	3345±454	3341±542	3190±580	NS

[1] (n) mean ± SD

[2] p - level of statistical significance by one-way analysis of variance

NS - not significant

TABLE 2

Relationship between maternal protein and energy intakes, change in maternal haemoglobin concentration (antepartum-initial) and birth weight

	Change in Haemoglobin (g/dl)						
Parameter	<-1.0	-1.0-<-0.5	-0.5-<0.0	0.0-<0.5	0.5-<1.0	>1.0	p[2]
Patient numbers	258	165	203	197	161	128	
Pregravid wt (kg)	58.1±11.5[1]	58.6±12.5	57.8±12.1	57.0±12.0	55.5± 9.8	54.8± 9.6	<0.025
Protein intake[3] ($g \times 10^3$)	6.0± 5.0	5.1± 4.6	5.1± 4.2	5.3± 4.5	4.2± 4.5	4.3± 3.8	<0.01
Energy intake[3] ($kcal \times 10^3$)	104.1±129.0	88.0±120.3	87.5±110.2	93.2±105.9	72.1±111.1	68.2±87.9	<0.07
Birth weight (g)	3397±511	3391±467	3328±462	3350±505	3270±479	3250±488	<0.025

[1] (n) mean ± SD

[2] p - level of statistical significance by one-way analysis of variance

[3] The large SD in the dietary data are due to the wide range in time of registration for antenatal care and hence a wide range in length of nutritional rehabilitation. The protein and energy intakes are in increases in intake during pregnancy under the Diet Dispensary service.

TABLE 3

Relationship between late antepartum haemoglobin concentration and infant birth weight

	Late Antepartum Haemoglobin (g/dl)						
Parameter	<10.0	10.0-10.9	11.0-11.9	12.0-12.9	13.0-13.9	>14.0	p[1]
Patient numbers	31	165	383	367	142	24	
Antepartum Hb (g/dl)	9.5±0.4[2]	10.5±0.3	11.5±0.3	12.4±0.3	13.3±0.3	14.3±0.4	<0.001
Birth weight (g)	3424±454	3432±495	3344±489	3338±442	3249±561	3105±602	<0.01

p - level of statistical significance by one-way analysis of variance

Mean ± SD

a failure of adequate plasma volume expansion or to an increase in total red cell mass. The present study does not permit a differentiation between the 2 causes. However, available data on changes in plasma volume and red cell mass would support the view that high antepartum haemoglobin is due to a failure of adequate plasma volume expansion (1).

Singla et al (14), in studying the effect of iron deficiency anaemia on placental and infant birth weights, showed a reduction in infant birth weight with an antepartum haemoglobin <11 g/dl. Garn and co-workers (16) showed an increase in adverse pregnancy outcomes with antepartum haemoglobin <11 g/dl for white women and haemoglobin <10 g/dl for black women. Folate deficiency may also cause anaemia during pregnancy and is associated with increased foetal wastage (4). Iron deficiency anaemia is associated with microcytic hypochromic cells (i.e. reduced MCV and MCH), whereas folate deficiency is associated with an increase in MCV with a normal MCH (9). Since the present study was retrospective as far as the haematological aspects were concerned, it was not possible to assess more directly the presence of iron or folic acid deficiency. However, taken as a whole, 40% of mothers with an initial haemoglobin <11 g/dl had reduced MCV and MCH, supporting a diagnosis of iron deficiency. It is gratifying to note that with the Montreal Diet Dispensary's nutritional rehabilitation program (including iron and folate supplements), these women's haematological status improved. Furthermore, the birth weights of their infants were not lower than those of the other groups of women with more normal initial haemoglobins.

It has been suggested that 11 g/dl is the lower limit of normal for maternal haemoglobin at or near term in women receiving iron supplementation (2). In the present study, 17.6% (196) of mothers had a haemoglobin <11 g/dl and, furthermore, these mothers also had among the largest babies (Table 3). Pritchard (2) points out that although a haemoglobin of 10-11.0 g/dl may be due to haemodilution, a haemoglobin <10 g/dl is a strong indication of a haematological abnormality. Of the 31 mothers with haemoglobin <10 g/dl (mean 9.5±0.4 g/dl) we had red cell indices for only 8. They had significantly lower MCV (79.6±8.7 fl) and MCH (26±3.4 pg) than those with higher haemoglobins. Presumably these women were still iron deficient and the supplement of 65.75 mg iron was insufficient to meet their haematopoietic needs. Nonetheless, these women had large infants. In all, 17 women were shown to have an antepartum MCV<80.8 fl or an MCH<26.4 pg. Their mean change in haemoglobin was -0.8 g±1.1 and their infants' mean birth weight was 3456 g±612. Our results would suggest that the major factor influencing birth weight in the studies of Singla et al (14) and Garn et al (16) was an inadequate food intake (protein and energy) rather than iron deficiency per se. Furthermore, our data show an inverse relationship between change in haemoglobin and additional amounts of protein taken during pregnancy (Table 2). For increases in energy there is also a clear gradient in mean values, but the high variance (due to the wide range in the time of registration and thus length of nutritional treatment) prevented attaining a conventional significance level ($p<0.07$). These data are consistent with the effect of protein and total food intake on plasma volume expansion in pregnant rats (6) mentioned above. It appears that the decrease in haemoglobin seen with the increased protein intakes was attributable to plasma volume expansion which in turn was associated with an increased infant birth weight.

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FOOTNOTE

Tables giving details of maternal characteristics and haematological indices are available on request from Dr. Pencharz.

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THE EFFECTS OF 2% MILK INTAKE ON INFANT NUTRITION

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ABSTRACT

Defatted milks are not recommended during the first year of life. Despite this, by 6 months of age many parents feed their infants 2% milk. The effects of 2% milk intake on the nutrient intake and growth of 6 to 12 month-old infants who participated in a longitudinal nutrition survey conducted in Toronto and Montreal were examined. Infants given a 2% milk diet ate significantly more food, protein and calcium, phosphorus, sodium, potassium, and riboflavin but less fat than infants fed formula or whole cow's milk. Energy provided by protein was significantly higher and fat lower in the 2% milk diet. The renal solute load in the 2% milk diet was significantly higher than in the whole milk diets. Despite these dietary differences, physical size and growth between the 2 groups of infants were similar. The implications of the results are discussed. Further studies are suggested to resolve the validity of current recommendation.

KEY WORDS: Infant nutrition, Growth, Milk

INTRODUCTION

The importance of milk as a food for infant feeding is well recognized. Milk is the sole food given to very young infants and remains one of the primary food sources during the first 2 years of life (1). The Canadian Pediatric Society (2) and Fomon et al (3) recommend that infants should be started on breast milk, or alternatively formula. Cow's milk is not recommended before 6 months. The use of defatted milks, such as skim and partially skimmed milk, is not advised until the infant is at least 1 year old. The latter recommendation against the use of defatted milk is related to its low fat content, which could result in a low level of dietary essential fatty acids. The high protein content is also considered undesirable. In the studies by Fomon et al (4) less than 6-month-old infants who were fed skim milk were found to receive significantly less dietary energy and fat, but more protein than infants fed regular formula. The skim milk fed infants were found to have slower weight gain, but faster gain in length. They also had less subcutaneous fat. This suggested that skim milk fed infants had to mobilize body fat to maintain needed energy. Whether or not infants fed partially skimmed (2%) milk behave similarly has not been reported.

We reported earlier that there was a resurgence in breastfeeding in Canada, both in initiation and duration (5). Despite this, feeding defatted milks, especially 2% milk became prevalent by 6 months, when over 50% of the infants received defatted milks. By eight months over 70% of the infants were fed defatted milks of which most was 2% milk. Hence many doctors and parents were not complying with the recommendation to not feed defatted milk during the first year of life.

During the period from 6 to 12 months of age, the mean daily fat intake of the Toronto and Montreal infants contributed less than 30% of dietary energy (1), a level that is considered undesirably low (6). Skim and 2% milk were implicated as the principal reason for this observation. This paper further examines the effects of 2% milk feeding on nutrient intakes, body size and growth of 6 and 12-month-old infants.

METHODS

Between 1977 and 1979 we conducted a longitudinal survey of infant nutrition in Toronto and Montreal. The design of this study was described in a paper published earlier in this journal (1). In brief, the dietary intakes of the infants, which initially consisted of 403 subjects were estimated by the 4-day dietary record at 1, 3, 5, 6, 7, 8, 10, 12, 15, and 18 months. The nutrient intakes were estimated using a modified version of the Nutrition Canada Nutrient Data Tape updated with current food composition values. Birth weight and length were determined by the hospital staff. At nine of the 10 study intervals, physical size of the infants was determined by weight, length, head circumference, arm circumference, and triceps and subscapular fatfolds (7). No measurements were taken at 7 months. All measurements were taken as described by Foman (8) by trained personnel.

In this paper only data obtained at 6, 8, 10 and 12 months are presented; the 7th month is omitted because of the lack of physical measurements. The survey population sizes at the four study intervals are shown in Table 1.

TABLE 1

Population Size

Age (month)	Total Survey Population	Group 1	Group 2
6	356*	123	100
8	349	92	149
10	341	81	173
12	333	74	178

* Number of infants

Group 1 consisted of infants whose milk intakes were $\geq$ 90% two percent milk by weight.
Group 2 consisted of infants whose milk intakes were $\geq$ 90% regular formula or whole cow's milk (NDM) by weight.

TABLE 2

Mean Food and Nutrient Intakes of Infants Fed 2% and Non-defatted Milks

	6 months		8 months		10 months		12 months	
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
Amount of Food (g)	1139*	1016	1197**	1103	1225	1184	1218	1207
Energy (kcal)	713	714	800	827	877	898	922	950
Protein (g)	35.1*	22.2	38.0*	31.4	40.6*	35.5	42.1**	38.6
Fat (g)	19.3*	28.9	22.0*	30.5	26.2*	32.5	29.5*	36.0
Carbohydrate (g)	99.6**	91.7	112.9	107.7	120.9	117.3	122.9	120.1
Calcium (mg)	1098*	727	1109*	848	1045*	853	986*	850
Phosphorous (mg)	939*	607	992*	763	875*	818	960*	842
Iron (mg)	12.2	12.3	12.2	12.7	11.0	10.1	9.7	8.1
Sodium (mg)	588*	323	671*	501	850*	679	938	888
Potassium (mg)	1800*	966	1874*	1369	1925*	1565	1921*	1678
Vitamin A (IU)	6402	6287	6441**	7512	6626	7005	5753*	7348
Thiamin (mg)	0.77	0.75	0.81	0.80	0.78	0.73	0.73	0.73
Riboflavin (mg)	1.91*	1.30	1.98*	1.60	1.89*	1.60	1.76**	1.61
Niacin (mg)	7.73	8.23	9.21	9.49	9.99	9.62	10.41	10.41
Vitamin C (mg)	73.2	74.6	78.0	86.0	90.7	93.3	97.4	101.6
Caloric Density (cal/100 gm.)	62.9*	70.6	67.4*	75.2	72.2**	75.9	76.8	79.0

* significantly different from Group 2 at $P < 0.001$.
** significantly different from Group 2 at $P < 0.05$.

For the present purpose, infants who were included in either milk group were infants whose milk intakes were 90% or more by weight of the respective types of milk. Non-defatted milks (NDM) included both regular forumla and/or whole cow's milk. Infants who were not thus classified were excluded. Furthermore, since we did not measure the volume of breast milk ingested, breast-fed infants were also excluded. Group 1 consisted of infants who were fed mainly 2% milk and group 2, infants fed mainly NDM.

Comparison of the growth rates of the two groups of infants was conducted only among those infants who were consistently fed either 2% milk or NDM from 6 to 12 months of age. This criteria for sample selection explains the small sample sizes shown in Table 7.

Statistical analysis of difference between means was by the Student's t-test (9).

RESULTS

Group 1 ate significantly more food than Group 2 at 6 and 8 months but not at 10 and 12 months (Table 2). Energy intake of the two groups of infants was similar. During all the 4 months, group 1 consumed significantly more protein, calcium, phosphorus, sodium, potassium and riboflavin, but less fat than group 2. Differences in carbohydrate, iron, thiamin, niacin, and vitamin C were not significant. Vitamin A intake of the two groups at 6 months was similar. However at 8, 10 and 12 months, the vitamin A intake of Group 1 was less than Group 2. Despite the observed differences, the mean nutrient intake of the two groups of infants were above the recommended daily nutrient intake of the Canadian Dietary Standard (10). This suggested that the diets of the infants were nutritionally adequate at least with respect to the protein, vitamins, and minerals considered in the present study.

The mean milk and non-milk food consumption patterns of the infants are shown in Table 3. The significantly greater food consumption of group 1 at 6 and 8 months was due to the apparent greater consumption of both milk and non-milk products. At the latter months, differences in the amount of food consumed by the 2 groups of infants were not statistically significant. Group 1 tended to consume more milk but less non-milk products than group 2.

TABLE 3

Mean Amount of Food and Milk Consumed by Infants Fed 2% and Non-defatted Milks

Age (month)	Total food (g)		Volume of Milk (ml)		Amount of Non-milk Foods (g)	
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
6	1139*	1016	702	699	437*	317
8	1197**	1103	676	624	522	479
10	1225	1184	619	571	606	613
12	1218	1207	579	538	639	668

* significantly different from group 2 at $P < 0.001$.
** significantly different from group 2 at $P < 0.05$.

The mean energy sources of the diets are shown in Table 4. In the diets of group 1, protein contributed between 18 and 20% of the dietary energy, fat 24 to 29% and carbohydrate 53 to 56%. For group 2 protein provided 12 to 16% of the dietary energy, fat 33 to 35% and carbohydrate 51 to 52%.

TABLE 4

Mean Energy Sources of Diets of Infants Fed 2% and Non-defatted Milks

Age (month)	Group 1			Group 2		
	Protein	Fat	Carbohydrate	Protein	Fat	Carbohydrate
	% dietary energy					
6	20	24	56	12	36	51
8	19	25	56	15	33	52
10	18	27	55	16	33	52
12	18	29	53	16	34	51

The dietary renal solute loads were calculated according to the formula described by Fomon (8). However, since the chloride contents of the diets were not determined because of the lack of food composition values, the results shown in Table 5 were underestimated. Nevertheless, the results show that the renal solute loads in the diets consumed by group 1 were significantly higher than that in group 2.

TABLE 5

Dietary Renal Solute Loads* of Infants Fed 2% and Non-defatted Milks

Age (month)	Group 1	Group 2	t	Significance
	mOsmol			
6	212 ± 42	127 ± 51	13.27	<0.001
8	229 ± 47	182 ± 70	6.19	<0.001
10	248 ± 59	211 ± 63	4.53	<0.001
12	258 ± 67	235 ± 78	2.37	0.019

Data are shown as Mean ± S.D.

* 1 g. protein = 4 mOsmol
1 meq. sodium = 1 mOsmol
1 meq. potassium = ImOsmol (8)

TABLE 6

Mean Physical Measurements of Infants Fed 2% Milk and Non-defatted Milks

Measurements	6 months		8 months		10 months		12 months	
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
Length (cm)	67.2	67.5	70.1	70.5	72.8	73.4	75.3	75.6
Weight (kg)	7.6	7.8	8.4	8.5	9.1	9.1	9.7	9.5
Head Circ. (cm)	43.6	43.6	44.9	44.8	46.0	45.8	46.8	46.4
Arm Circ. (cm)	14.2*	14.6	14.7	14.9	15.1	15.0	15.3	15.2
Triceps Fatfold (mm)	8.4	9.1	9.0	9.3	9.3	9.2	9.4	9.8
Subscapular Fatfold (mm)	6.8*	7.5	7.1*	7.7	7.3	7.3	7.2	6.9
Weight/Length (kg/cm)	0.11	0.11	0.12	0.12	0.13	0.12	0.13	0.13

* significantly different from Group 2 at $P < 0.05$

At all four study months, the mean weight and length of both groups of infants were within the normal ranges of the National Center for Health Statistics values (11). At no time were the differences in weight, length, head circumference, triceps fatfold and the weight over length ratio between the two groups of infants statistically significant (Table 6). The arm circumference of group 1 was slightly but significantly smaller than group 2 at 6 months but not in subsequent months. The subscapular fatfold of group 1 was significantly smaller than group 2 at 6 and 8 months but not at 10 and 12 months.

The growth rate of the two groups of infants was similar (Table 7). The gain in weight, length and head circumference of infants who were consistently fed either 2% milk or NDM from 6 to 12 months were not significantly different.

TABLE 7

Growth of Infants Fed 2% and Non-defatted Milks Between 6 and 12 Months of Life*

Gain in	Group 1		Group 2		t	Significance
	N		N			
Length (cm)	67	8.16 ± 1.41	43	8.13 ± 1.58	0.12	0.906
Weight (kg)	68	2.06 ± 0.57	43	1.98 ± 0.56	0.73	0.464
Head Circumference (cm)	68	3.09 ± 0.67	43	3.00 ± 0.71	0.69	0.489

* Infants fed exclusively 2% milk or NDM from 6 to 12 months

DISCUSSION

Compared to whole cow's milk or formula, skim and 2% milk have proportionally more protein and less fat and energy. The Canadian Pediatric Society (2) and Fomon et al (3) advise against feeding defatted milks during the first year of life because of possible adverse nutritional effects. Indeed less than 6-month-old infants who were fed skim milk were found to eat more food but inadequate amount of energy (4). They were also found to experience stunted growth and reduced subcutaneous fat (4). However, no similar studies on infants fed 2% milk have been reported.

In the present study, 6 to 12 month old infants who were fed mainly 2% milk consumed more food than infants who were provided whole cow's milk or formula but their energy intake was the same as the latter. Thus it appears that the 2% fat in the milk and the compensatory greater food intake as milk, non-milk products or both was sufficient to raise the dietary energy content of the 2% milk diet to the level of the NDM diet. The present observations support the notion that infants eat according to their energy needs and not by the volume of food (8). However, the data do not support the idea that feeding 2% milk reduces or limits energy intake.

On the whole, both the 2% milk and NDM diets provided enough nutrients recommended by the Canadian Dietary Standard. However, the fat, protein and renal solute contents in the 2% milk diet might be considered inappropriate. According to Fomon (6) broad clinical experience suggested that the infants' diet should contain 7 to 16% of dietary energy from protein, 35 to 55% from fat

and the rest from carbohydrate. Clearly, results in the present study show that the 2% milk diet supplied more than the suggested range of energy from protein and less from fat; whereas, the NDM diet supplied proportionally more appropriate ranges from the two nutrients. The relatively low level of fat in the 2% milk diet is considered undesirable because of the potential inadequacy of essential fatty acid. The comparatively high level of protein in the 2% milk diet is considered inappropriate because of its potential risk of pre-renal azotemia and a hyperosmolar state (2). Indeed, the greater protein and electrolyte contents in the 2% milk diet contributed to the significantly greater renal solute load than in the NDM diet.

Despite the theoretical and potential dietary risks, growth of infants fed 2% milk was within the normal range and similar to infants fed the NDM diet. Differences in weight, length, head circumference and rate of growth between the two groups of infants were not statistically significant. However, at 6 months but not in subsequent months NDM infants had more subcutaneous fat than the 2% milk infants because of their larger arm circumference and subscapular fatfold. The latter observation suggests that young infants (6 months of age or less) may be more susceptible to dietary changes than older infants. Fomon previously observed that less-than-6 month old infants who were fed skim milk grew at a significantly slower rate than infants fed regular formula (4). Additionally they had less subcutaneous fat. The discrepancies in observations between the present study and Fomon's are attributable not only to differences in ages of the infants but probably also to the difference in fat content of skim and 2% milk.

Judging from the results of the physical measurements, the 2% milk diet does not appear to adversely affect growth of the infants. For this reason the recommendation to avoid feeding 2% milk to 6 to 12-month-old infants may be questioned. Theoretically, the 2% milk diet is deficient in essential fatty acids. However, whether or not the level of fat intake among infants fed the 2% milk diet produced a risk of essential fatty acid deficiency, remains to be determined. Fatty acid deficiency among full-term infants is rarely reported in the literature. If 2% milk diets are indeed low in essential fatty acids, reports of related nutritional problems would not be as scarce since 2% milk is commonly the milk fed infants after 6 months of life (5).

The lack of reported cases of essential fatty acid deficiency among infants may be due to an overestimation of requirement. Cutbertson (12) reviewed the subject and concluded that the minimum essential fatty acid requirement of 1% of dietary energy was probably set too high; the minimum requirement was more likely 0.5% of energy or half the currently recommended level. Whole cow's milk provides 0.6% to 0.8% of total energy from linoleic acid and 2% milk provides 0.3 to 0.4% (12). Thus whole cow's milk provides adequate amounts of linoleic acid but 2% milk is marginally low. The latter can be easily supplemented by other food sources. Unfortunately, in the present survey we were not able to estimate the dietary essential fatty acid content because of the lack of information in the food composition tables used. Further studies on the adequacy of essential fatty acid in the 2% milk diet are strongly encouraged.

Similarly, although the renal solute load in the 2% milk diet is significantly greater than in the NDM diet, its biological impact is not known. The renal solute loads in the 2% milk diet were within safe limits (8). The calculated renal solute loads per 1000 g of the 2% milk diet were 186, 191, 202, 212 mosmol at 6, 8, 10 and 12 months respectively. At these levels plasma osmolality may not be affected. Although infants of similar ages and diets have not been similarly studied, Dale et al (13) and Marlin, Picciano and Livant (14) reported no differences in plasma osmolality among 2 to 12 week old infants fed formula and breast milk despite the much greater renal solute load in the formula.

The lack of biochemical assessment and of developmental achievement of the infants in the present survey prevents any definitive conclusion to the appropriateness of 2% milk for 6 to 12 month old infants. Further studies are required to resolve the issue. Until then it remains prudent to follow the suggested guideline of avoiding defatted milks, including 2% milk, in diets of infants less than 1 year old.

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COMPARISON OF CALORIC AND NUTRIENT INTAKE OF ADULTS DURING WEEK AND WEEK-END DAYS

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ABSTRACT

Three-day food records in 174 women and 185 men were used to compare caloric and nutrient intake of week and week-end days. Both women and men were found to consume significantly more energy and a higher level of alcohol during the week-end days than during the week days. In women, a significant decrease in calcium intake was observed during the week-end days in the 36-40 and the 41-45 age groups, respectively. In general, the significant increase in energy intake did not correspond to a significant increase in other nutrients.

KEY WORDS: adults, nutrient intake, week, week-end.

INTRODUCTION

This study is a part of a multidisciplinary research program designed to determine the effects of heredity and environmental factors on physical fitness, performance and dietary behaviour. In previous studies, energy and nutrient intake of a subsample of adults (1) and adolescents (2) has been described. The purpose of the present study is to define the relationship between a dietary profile of 359 adults during the week days to that obtained during the week-end days, with respect to caloric and nutrient intake. As few recent dietary surveys have been conducted with healthy subjects, the present data will be compared to findings of the Nutrition Canada Survey (3) and the Canadian Dietary Standards (4).

METHODS

The French Canadian families participating in the present study were recruited by mass media and met certain selection criteria (1, 2). A total of 174 women and 185 men, aged from 36 to 50 years inclusive, completed a 3-day measured food record including one week-end day (Saturday, Sunday or holiday) and collected between June '78 and August '80. Records contained information about the quantity (weight or volume) of all foods consumed, the methods of food preparations, the period of the day when the food was eaten, the place where the food was eaten and the origin of foods (fast food, home-made, etc.). Families were met two times: first, by the research supervisor, to receive a metric scale (Hanson Dietetic Scale, Model 1440, Skikuta, Mississipi, U.S.A.), a glass measuring cup, a set of measuring spoons, a ruler and instructions to complete their record and second, by a trained dietary technician, to review and complete each dietary record with the subjects with the help of foods models, pictures and forms as required. Records were coded by two dietary technicians using the food composition table of Brault-Dubuc and Caron-Lahaie (5) based upon the corresponding American

tables (6, 7) and containing manufacturer's information, new foods and other nutrients such as alcohol and concentrated sugars. Ten percent of records were selected by a randomized method (8) and systematically recoded. The percentage of error was 0.02%. Week and week-end means for nutrient intake of energy, protein, fat, total carbohydrate, concentrated sugar, alcohol, calcium, iron, retinol, thiamine, riboflavin, niacin and ascorbic acid were obtained by data processing. Concentrated sugars included the refined and processed sugars that were added to foods.

Socio-economic variables, including family income and educational level, were obtained by questionnaire. Family income was indexed according to information from Statistics Canada.

Statistical analysis

The t-Test for paired data (9) was used to compare the mean caloric and nutrient intake during the week days and the week-end days according to sex and age groups (36-40, 41-45, 46-50 years).

RESULTS

Women and men were found to consume more calories ($p < 0.05$, $p < 0.001$) and a higher level of alcohol ($p < 0.001$) during the week-end days than the week days (Table 1). In women, a lower calcium intake was noted during the week-end days ($p < 0.001$) whereas all other nutrients were found equivalent to the week-day intake (Table 1). Tables 2 and 3 present mean energy and nutrient intake by age groups and sex. Regardless the age group, women consumed significantly more alcohol (twice as much) during the week-end days ($p < 0.001$, $p < 0.01$) than during the week days (Table 2). While caloric intake increased during the week-end for all age groups, the noted increase was significant only in the 41-45 age group. In this same group, an increase in the intake of concentrated sugars and a decrease in calcium were observed ($p < 0.05$, $p < 0.001$) (Table 2).

Men in all age groups consumed at least 1½ time more alcohol during the week-end days ($p < 0.001$, $p < 0.05$) than during the week days (Table 3). As noted for women, caloric intake of men increased significantly during the week-end days in the 41-45 age group and more significant differences ($p < 0.05$) were noted for the intake of protein, lipid and thiamine in this middle-age group. In the oldest age group (46-50), a significant decrease in the retinol and the riboflavin intake ($p < 0.01$, $p < 0.05$) were noted during the week-end days (Table 3).

DISCUSSION

The family income and educational level indicated a higher socio-economic status for the present sample than for the whole population of Quebec. In the present study, the indexed mean family income was $42,057 and 49% of the families had a no-indexed income above $30,000 as compared to 19% respectively for the whole population of Quebec (10). Moreover, 47% of men and 25% of women had obtained at least a junior college degree as compared to 25% and 19% respectively for the whole population of Quebec (11, 12).

The present findings demonstrate that the significant increase in energy intake observed in women and men seems to be related to a significant increase in alcohol consumption. In this respect, the present data reveal that in women and in men, alcohol intake provides 49 and 161 kcal and 112 and 301 kcal during the week days and week-end days respectively.

An intake of 43 g of pure alcohol is equivalent to the amount of alcohol contained in 4 bottles of beer, or in 14 ounces (398 ml) of wine, or in 4.5 ounces (128 ml) of dry gin.

TABLE 1

Mean nutrient intake during week and week-end days in women and men (Mean ± S.E.M.)

Nutrients		Women n=174		Men n=185	
		Week	Week-end	Week	Week-end
Energy	(kcal)	1822±34	1941±51*	2572±41	2766±66***
	(kj)	7614±141	8113±213*	10749±173	11561±276***
Protein	(g)	72±2	73±2	97±2	99±2
Lipid	(g)	75±2	80±3	103±2	108±4
Carbohydrate	(g)	208±4	208±6	282±5	286±7
Concentrated sugars	(g)	60±2	65±4	85±3	86±5
Alcohol	(g)	7±1	16±2***	23±2	43±4***
Calcium	(mg)	718±24	611±24***	860±29	863±35
Iron	(mg)	11.8±0.3	12.5±0.4	15.7±0.3	15.8±0.5
Retinol	(RE)	1266±110	1155±154	1440±115	1218±87
Thiamine	(mg)	0.97±0.03	1.04±0.05	1.25±0.03	1.32±0.04
Riboflavin	(mg)	1.59±0.05	1.51±0.08	2.03±0.06	2.02±0.06
Niacin	(NE)	18.4±0.4	19.3±0.6	25.4±0.6	26.1±0.7
Ascorbic acid	(mg)	88±3	98±6	96±5	100±5

* $p < 0.05$, *** $p < 0.001$

TABLE 2

Mean nutrient intake during week and week-end days in women by age groups (Mean ± S.E.M.)

Nutrients \ Age groups		36-40 n=78		41-45 n=64		46-50 n=32	
		Week	Week-end	Week	Week-end	Week	Week-end
Energy	(kcal)	1867±51	1966±86	1832±54	1997±73*	1693±80	1769±104
	(kj)	7800±212	8217±361	7657±224	8346±307*	7076±336	7392±434
Protein	(g)	74±2	77±3	72±3	71±3	66±4	65±4
Lipid	(g)	76±2	80±5	78±3	83±4	67±4	73±5
Carbohydrate	(g)	214±7	208±10	205±6	216±9	200±11	194±13
Concentrated sugars	(g)	61±4	61±6	58±3	69±6*	64±7	70±7
Alcohol	(g)	7±2	17±3***	6±2	17±3***	6±3	12±4**
Calcium	(mg)	733±37	638±39*	715±37	569±35***	688±53	626±51
Iron	(mg)	12.2±0.4	13.3±0.8	11.8±0.5	12.8±0.5	11.0±0.6	10.2±0.7
Retinol	(RE)	1237±174	1304±265	1316±177	1134±263	1238±245	838±80
Thiamine	(mg)	0.98±0.11	1.07±0.12	1.00±0.13	1.08±0.14	0.88±0.05	0.89±0.07
Riboflavin	(mg)	1.56±0.07	1.61±0.16	1.68±0.21	1.51±0.19	1.48±0.12	1.27±0.10
Niacin	(NE)	19.3±0.7	20.5±1.1	18.2±0.7	19.3±0.8	16.9±0.7	16.2±1.2
Ascorbic acid	(mg)	82±5	96±9	93±5	104±9	89±9	92±12

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

TABLE 3

Mean nutrient intake during week and week-end days in men by age groups (Mean ± S.E.M.)

Nutrients \ Age groups		36-40 n=59		41-45 n=74		46-50 n=52	
		Week	Week-end	Week	Week-end	Week	Week-end
Energy	(kcal)	2648±63	2781±104	2558±60	2913±110***	2505±92	2540±126
	(kj)	11068±263	11621±435	10690±251	12177±460***	10472±406	10615±527
Protein	(g)	99±3	100±4	96±3	104±4*	94±4	91±4
Lipid	(g)	108±4	108±5	100±4	113±6*	101±5	100±7
Carbohydrate	(g)	291±9	287±11	283±8	301±12	270±10	264±14
Concentrated sugars	(g)	92±6	81±8	83±5	88±7	81±6	90±9
Alcohol	(g)	20±3	42±7***	25±4	48±6***	24±5	37±7*
Calcium	(mg)	908±56	908±71	872±49	878±55	789±43	791±57
Iron	(mg)	16.2±0.7	16.8±1.0	15.4±0.5	16.0±0.6	15.6±0.7	14.4±0.8
Retinol	(RE)	1298±150	1387±201	1393±192	1274±136	1668±255	948±70**
Thiamine	(mg)	1.28±0.06	1.31±0.07	1.23±0.04	1.42±0.07*	1.26±0.06	1.18±0.07
Riboflavin	(mg)	2.03±0.11	2.10±0.3	2.01±0.09	2.09±0.10	2.07±0.13	1.82±0.10*
Niacin	(NE)	25.4±1.0	26.5±1.3	25.3±0.9	27.0±1.2	25.4±1.33	24.2±1.40
Ascorbic acid	(mg)	88±9	97±9	103±7	113±8	94±7	86±7

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

In terms of absolute quantities of alcohol, the intake is almost three times higher during the week-end days in both women and men as during the week days. Overall alcohol consumption is significantly higher in men than in women during the week-end days as well as during the week days (1; Table 1).

In the light of these findings, it seems that Nutrition Canada values (3) may have underestimated the real mean alcohol consumption. In fact, a 24h dietary recall was used, as it is currently employed in such National Surveys (13) and the alcohol intake was not collected during the week-end days. It has been recognized that a 24h dietary recall tends to underestimate mean intakes (14) whereas the accuracy of a 7-day dietary record declines with the fourth day of record when the motivation of subjects is decreasing (15). In such cases, populations having a low socio-economic status did not perform adequately (13, 15) and are under-represented as it was observed in the present study. Moreover, the present data indicate that the alcohol intake during the week days, 49 and 112 kcal for women and men, is higher than Nutrition Canada findings. In that study a 24 to 31 kcal intake was noted for women aged from 20 to 39 and from 40 to 64 years respectively, while a 120 to 138 kcal intake was reported for men aged from 20 to 39 and from 40 to 64 years respectively.

Statistics from government sources (Société des Alcools du Québec, unpublished data) indicate that the mean alcohol intake per day for the total Quebec population, including children, was of 123 kcal, as pure alcohol, in 1980-81. In the present sample, a mean daily alcohol intake of 140 kcal was observed with women and men grouped together. While the present findings seem to be in the same order of magnitude than official figures, it must be noted that government statistics represent a per capita mean consumption figure. Our data apply to a specific age group, represented but not specifically identifiable in governement findings.

In women, the increased alcohol intake was accompanied by a significant decrease in calcium intake (Table 1). Mean calcium intake surpasses the Canadian Dietary Standards (CDS) (4) during the week days but decreases to 87% of the CDS during the week-end days. The decreased calcium intake observed during the week-end days, particularly in the middle age group, reflects variations in food consumption pattern. It is possible that less milk and other dairy products are consumed while more alcohol is ingested. Certain meals may be skipped giving way to larger combined meals (brunch) or a snacking-type of meal pattern may be adopted.

In men age from 41 to 45 years, the significant increase in protein, lipid and thiamine may indicate a higher consumption of delicatessen-type meat during the week-end days compared to meals with whole meat in sandwiches or combination foods such as casseroles, stews, etc. Unfortunately, the present data are represented only in terms of mean intake of nutrients. Proposed changes in the data processing system will permit us to identify food patterns or intake models of the type applied recently to large population studies (16). Such models are useful to identify groups of foods preferentially chosen or rejected according to varying circumstances. Only when specific foods have been identified with week or week-end eating behavior can appropriate nutrition education be attempted.

CONCLUSIONS

The present study reveals that the dietary profile of 359 healthy adults varies significantly during the week-end days with respect to the intake of energy and alcohol in both sexes. The study underlines the importance of including a week-end day to increase the accuracy of the estimation of mean individual nutrient intake when using multiple day food records or diaries.

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SODIUM AND POTASSIUM CONTENT OF THE CANADIAN DIET

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ABSTRACT

On the basis of the food consumption data obtained in the Canadian Nutrition Survey, representative food samples were collected in Vancouver, Winnipeg, Montreal and Halifax during summer and winter. After preparing the foods in a normal way, but without adding salt, ten food group composites were prepared. They were analysed for sodium and potassium. The total diet was found to contain 1142 mg sodium and 1194 mg potassium per 1000 kcal. The sodium included that added by the food manufacturing industry to about one-quarter of the 120 food items included in the diet. About 70 percent of the sodium and 50 percent of the potassium were provided by three major food groups viz. milk and dairy products; meat, fish and poultry; cereals and cereal products. Fruit products, vegetables, potatoes and mixed dishes supplied 9-16 percent of the potassium. Vegetables and mixed dishes contained about 10 percent of the sodium. The amounts of potassium in the diets of various physiological groups, including pregnant women, were more than the Canadian recommendations. The sodium intakes were 2 to 5 times the recommended amounts.

KEY WORDS: Sodium, Potassium, Diet, Canada

INTRODUCTION

In recent years dietary salt has attracted a great deal of attention because it has been implicated in the widespread incidence of hypertension in Japan and many Western countries. In Canada a committee on diet and cardiovascular disease (1) recommended a reduction of salt intake. A similar recommendation was later made by a U.S. Senate Committee (2). Although the levels and sources of sodium and potassium in U.S. (3) and British (4,5) diets have been recently determined, similar data on the Canadian diet are not available. Two decades ago urinary excretion of sodium and potassium by Canadian adults was reported to average 162.6 and 66.1 mEq per 24 hr, respectively (6). With a view to determine the dietary levels of sodium and potassium it was decided to analyse representative Canadian diets.

MATERIALS AND METHODS

Food Groups

The Food Directorate, Health Protection Branch, Health and Welfare, Canada, instituted a Total Diet Study for monitoring pesticide residues (7). In this project food samples were collected in April 1974 to February 1975 from Halifax, Montreal, Winnipeg and Vancouver. The details of the foods and amounts were identical to those described by McLeod et al. (7). In brief, the food consumption data obtained in the Canadian Nutrition Survey (8) were used to select 120 foods consumed in significant amounts. Using the population distribution in Canada, and the daily consumption of each food by individuals in different age and sex groups, per capita consumption of each food was calculated. Suitable amounts of each food were collected in four cities. The individual foods were prepared as in a home, and then items included in the different food groups (e.g. dairy products; meat, fish, poultry, eggs; cereals) were homogenized in a blender. No salt was added to any food in cooking. The homogenized samples of each food group, collected in two seasons (Summer - April to September, Winter - October to February) in the four cities were stored in a freezer at -20°C. A total of 78 samples were prepared and stored in this way.

Sample Preparation and Analysis

Three to five grams of each sample were weighed into 50 ml silica beakers in duplicate, dried overnight in an oven at 105°C, and then ashed in a muffle furnace at 450°C using concentrated nitric acid as an oxidizing agent to obtain white ash. The ash was dissolved in 4 ml of 25% concentrated hydrochloric acid and diluted to 30 ml. The solution was analysed for sodium and potassium by atomic absorption spectroscopy. In the analysis of food groups containing garden fruits and fruit products, caution had to be exercised in ashing because of the high potassium content. The temperature of the muffle furnace was controlled at 375°C (after the addition of concentrated nitric acid) instead of 450°C, since there is a chance of the loss of the sample due to a minor explosion above 375°C.

NBS Standard Reference Material 1577 Bovine Liver was analysed by this method for sodium and potassium. Results obtained were within the certified ranges.

RESULTS AND DISCUSSION

The amounts of the different foods (grouped into food categories) consumed daily on a per capita basis are given in Table 1. Examples of items included in each group and the proportion of calories provided by the food groups are also included in the table. About one quarter of all the items were processed by the food manufacturing industry to varying degrees. In Table 2 are shown the sodium and potassium levels, total daily intake and the percentage contribution of each group. The average amounts of foods of each group consumed daily by individuals in different age and sex groups are shown in Table 3. Using these data, the sodium and potassium contents were calculated and these are given in Table 4. The amounts of sodium and potassium per 1000 kcal are also included in the same table.

From the results in Table 2, it is evident that cereals and cereal products provide about one-third of the sodium in the Canadian diet. The proportion of sodium from cereals in the U.S. diet was comparable (3) but this group provided about 40 percent of the sodium in the British diet (4). Most of the sodium in the cereals group, naturally low in sodium, is added in the form of salt, baking powder or baking soda (9). The total sodium contents of the three diets are not directly comparable because of the differences in

TABLE 1
Food Intake Per Person Per Day

	Food group[a]	Examples of items included	Amount, g	% Calories[b]
1.	Dairy products	Fluid and dried milk, buttermilk, yoghurt, cheese, ice cream.	426	15
2.	Meat, poultry, fish, eggs	All varieties of meat, poultry, fish and eggs.	172	20
3.	Cereal products	Bread and rolls, pasta, grains and flours, breakfast cereals.	235	27
4.	Fruit and fruit products	Fruits - fresh, canned, frozen, dried, fruit juices, nectars, drinks.	232	6
5.	Vegetables	All types of vegetables.	117	8
6.	Potatoes	All types except sweet.	124	
7.	Fats	Butter, margarine, oils, shortening, peanut butter.	25	7
8.	Nuts and dried legumes	Peanuts, coconuts, dried peas, beans, soybeans.	12	
9.	Foods primarily sugar and sugar from beverages	Syrups, cake icings, honey, jam, jelly.	51	16
10.	Miscellaneous	Mixed dishes, soups, condiments.	118	

[a] As described in (8).

[b] Total kcal/person/day = 2400.

calorie content and the amount of salt added in the preparation of the foods. In the British diet salt was added as is normal in the preparation of foods but in the U.S. diet 1600 mg of sodium was added to the group "sugar and adjuncts" to allow for the discretionary use of salt by the consumer. In the Canadian diet, however, salt was not added in the preparation of the foods. Thus the amount of sodium per 1000 kcal of the British diet was 1416 mg compared to 1307 (excluding the discretionary amount of 1600 mg) and 1142 mg in the U.S. and Canadian diets respectively. Obviously the higher level of sodium in the British diet was partly due to the salt added in the preparation of the foods. Probably for the same reason, 85% of the sodium in the British diet came from the three major food groups viz. milk, dairy products; meat, fish, poultry; and cereal products; whereas the corresponding sodium contribution in the U.S. (excluding the discretionary amount of 1600 mg) and Canadian diets was about 70 percent.

Three major food groups, including dairy products, meat, fish, poultry and cereal products, provide less than 50 percent of the potassium in the Canadian diet. The corresponding contribution in the British and the U.S. diet was approximately 55 percent. The potassium content per 1000 kcal of the three diets was 1194 (Canadian), 1110 (British) and 1170 (U.S.), with, as expected, less variation than with sodium, which is added in processing of a variety of foods. Fruits, vegetables, potatoes and mixed dishes were significant contributors (9-16%) of potassium but of these only vegetables and mixed dishes provided 9-10 percent of the sodium in the Canadian diet.

TABLE 2
Sodium and Potassium in the Canadian Diet

Food group	Sodium			Potassium		
	mg/100 g[a]	mg/day	% of total	mg/100 g[a]	mg/day	% of total
1	104	442	16	153	651	23
2	333	574	21	232	399	14
3	386	906	33	125	293	10
4	2	5	-	188	437	15
5	227	266	10	225	263	9
6	42	53	2	371	461	16
7	697[b]	171	6	180	44	2
8	234	29	1	194	24	1
9	70	36	1	87	45	2
10	218	258	9	209	248	9
Total		2740			2865	
Per 1000 kcal[c]		1142			1194	

[a] Each result is the average of 8 to 16 determinations. The coefficient of variation for samples from different cities was 40% for sodium and 20% for potassium.

[b] Peanut butter included in sample.

[c] Total kcal/person/day = 2400.

TABLE 3
Intake of Foods in Different Groups (g/day)[a]

Food group	Age, yr										
	1-4	5-11	12-19		20-39		40-64		65+		Pregnant Women
			M	F	M	F	M	F	M	F	
1	685	630	711	473	420	289	332	225	273	255	544
2	89	116	197	131	285	160	211	145	162	102	160
3	149	251	326	216	282	198	269	174	252	183	202
4	258	312	237	237	244	204	194	239	165	208	301
5	56	83	94	97	155	134	134	136	118	103	156
6	75	110	185	115	189	99	131	79	124	80	114
7	11	21	34	23	33	22	29	20	24	16	24
8	6	31	20	15	15	8	15	10	8	5	15
9	36	51	65	50	64	44	56	39	56	40	50
10	96	118	140	127	132	106	150	89	108	82	116

[a] As given in (8).

TABLE 4
Sodium and Potassium Intake of Canadians

Age, yr	Sex	kcal	Sodium		Potassium	
			mg/day	mg/1000 kcal	mg/day	mg/1000 kcal
1-4	M,F	1666	2070	1242	2590	1555
5-11	M,F	2300	2720	1183	3080	1339
12-19	M	3251	3580	1101	3740	1150
	F	2243	2540	1132	2770	1235
20-39	M	3374	3510	1040	3580	1061
	F	2001	2380	1189	2430	1214
40-64	M	2671	3050	1142	2930	1097
	F	1726	2120	1228	2220	1286
65+	M	2056	2580	1255	2480	1206
	F	1530	1910	1248	2020	1320
Pregnant Women		2259	2780	1229	3150	1394

The results in Table 4 show that the minimum amounts of sodium and potassium per 1000 kcal occur in the diet of 20-39 year old males who have the highest caloric intake. The amount of potassium per 1000 kcal in the diet of 1-4 year old children was higher than in the diet of all other age groups probably because of the predominance of dairy and fruit products in their diet. It is also worth noting that the potassium level in the diet of pregnant women was much higher than in the diet of females of child bearing age. This was also a reflection of the increased consumption of dairy and fruit products (Table 3).

Compared with the recommended intakes of sodium for various physiological groups (10), the amounts in their diet were 2 to 5 fold; except the diet of 12-19 year old males and females, which contained slightly more than the recommended amounts of sodium. On the other hand, the diet of 1-4 year old children only, contained three times the recommended amount of potassium, whereas the diets of other groups, including pregnant women, provided amounts, which were sufficient to meet the recommendations (10).

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ENZYMATIC DIGESTION METHOD WITH DIALYSIS TO ASSESS PROTEIN DAMAGE: APPLICATION TO ALKALI-TREATED PROTEINS CONTAINING LYSINOALANINE [1,2,3]

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ABSTRACT

An in vitro method is proposed to study protein digestion and amino acid availability. It uses a pepsin-pancreatin proteolysis and continuous removal of digestion products by dialysis through a membrane to simulate absorption in the intestine and to prevent proteolysis inhibition. Digestion was evaluated by measuring the amount of dialyzable products consisting of a mixture of amino acids and small peptides. High extent of digestion of casein, soybean and rapeseed proteins was achieved in a relatively short period with this method; some amino acids were released rapidly, mainly basic and aromatic amino acids, while proline and dicarboxylic amino acids were released at slow rates. The proteins were submitted to heat and alkali treatment that induces the formation of lysinoalanine (LAL) at the expense of lysine. The in vitro method was used to assess the effect of this treatment on protein digestion. Heat and alkali treatment at 60° and 80°C reduced in vitro protein digestibility in the following decreasing order: casein > soybean protein > rapeseed protein. A maximum effect on protein digestion did not correspond to maximum change in the digestibility of specific amino acids. Lysinoalanine was poorly released by digestive enzymes except in severely treated casein (pH 12, 60° or 80°C for 4 h). This illustrates the nutritional consequences of processing and the possibility of using the in vitro method for protein quality evaluation.

KEY WORDS: Enzymatic in vitro digestion, dialysis, alkali treatment, lysinoalanine.

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[3] Scientific publication No 507 from the Food Research Institute, Ottawa, Canada.

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INTRODUCTION

Data on amino acid composition are indispensable for the evaluation of the nutritional quality of a protein source but they yield no information about the availability of the amino acids to the organism. Since the nutritional quality of a protein is related to both its amino acid content and the ability of digestive enzymes to liberate them, various methods using digestive enzymes have been proposed and several enzyme systems tried (1,2). Since it is most desirable that the method reproduces the sequence of digestion in the digestive tract, the principal limitation of most of the methods proposed is the inhibition of the proteolysis resulting from the accumulation of digestion products in the medium (3), and their suitability for estimating the extent of digestion. To overcome these problems, we devised a method based on the enzymatic hydrolysis of proteins with simultaneous dialysis of digested products, adapted from the original idea of Mauron et al. (4,5).

Heat treatment of food proteins under alkaline conditions is well established in food technology. It is used to prepare protein isolates, destroy undesirable components in proteins (toxins, enzymes, enzyme inhibitors, microorganisms, etc.) and alter the flavour or functional properties of proteins (6). The chemical changes brought about during this type of processing are of increasing concern to the nutritionist. The concerns are of two types. First, this processing may cause a decrease in nutritive value as a consequence of the reduction in bioavailability of essential amino acids by degradation, racemization, crosslinking and formation of amino acid-sugar complexes when sugars are available (4,6,7,8). Second, heat and alkali treatment has toxicological consequences in that it induces the formation of lysinoalanine (LAL). This compound is formed by the condensation of the ε-amino group of lysine with dehydroalanine residues produced from the β-elimination from cystine and serine elsewhere in the protein, resulting in crosslinks (8). Lysinoalanine induces renal lesions in the rat characterized by a nuclear and cytoplasmic enlargement of the epithelial cell layer in the "pars recta" of the proximal tubule (9,10). It is much more toxic given in the free form than when attached to the protein molecule probably because of its low digestibility thereby reducing its availability (10-14). This has created controversy about the real toxicity of protein bound lysinoalanine.

To achieve a better understanding of the nutritional effect of alkali treatment and of lysinoalanine availability to the organism, three proteins, casein, soybean and rapeseed proteins were heat-treated in the presence of alkali and digested enzymatically in vitro according to the above mentioned procedure.

MATERIALS AND METHODS

Chemicals

The following chemicals were used: acetonitrile and acetone, HPLC grade, distilled in glass (Fisher Scientific, Montréal, Québec), norleucine (British Drug House, Montréal, Québec), lysinoalanine-HCl (Miles Lab., Rexdale, Ontario), other amino acids, their derivatives and dansyl chloride (5-dimethyl-aminonaphtalene-1-sulfonyl chloride) (Sigma Chemical, Saint-Louis, Mo). The protein sources were casein (High Protein, ICN Nutritional Biochemicals, Cleveland, OH) a soybean protein isolate (Edible soy protein, Promine F®, Central Soya, Chicago, IL) and rapeseed protein concentrate (prepared by the FRI-71 process at the Food Research Institute, Agriculture Canada, Ottawa). Pepsin (hog stomach mucosa 1:20 000), pancreatin (hog pancreas 5 x) and thimerosal were purchased from ICN Nutritional Biochemicals.

Alkaline treatment

Samples of 50 g of protein were suspended in 450 ml 0.1 N NaOH in a beaker with the pH adjusted to the desired value with N HCl and continuously stirred in a controled temperature water bath at the prescribed conditions of temperature and duration. The treatment was stopped by the addition of 5 ml 6 N HCl and the suspension adjusted to pH 4.5. The suspension was centrifuged for 30 min at 2600 *g*. The residue was washed once with water (pH adjusted to 4.5), freeze dried and stored at 4°C for nitrogen determination and amino acid analysis. The washing procedure did not change the composition of the protein or its in vitro digestibility.

Nitrogen determination

Nitrogen content of samples was determined with the aid of an autoanalyser using the method No 329-74 W/B of Technicon, Tarrytown, N.Y.

In vitro digestion of proteins with simultaneous dialysis

The in vitro digestion method for proteins was a two-step proteolysis performed at 37°C. The protein (2.5 g:400 mg N x 6.25) was first digested with 100 mg pepsin (5 mg/ml 0.1 N HCl) in 100 ml 0.1 N HCl at pH 1.9 in a beaker for 30 min. The pepsin proteolysis was stopped by addition of 5 ml N NaOH and the suspension adjusted to pH 8.0. The digestion was continued for 24 h with 100 mg pancreatin (5 mg/ml phosphate buffer) in a cellulose dialysis membrane (27 cm long x 4.5 cm flat width) having a molecular weight cutoff of 1000 (Spectra/Por® 6 membranes, Spectrum Medical Industries Inc., Los Angeles, CA). The mixture was stirred by means of a flat and helicoidal glass rod. The dialysis bag was suspended in a U-shaped glass container (30 cm long x 5.2 cm i.d.) filled with 400 ml 0.1 M sodium phosphate buffer pH 8 and immersed in a constant temperature water bath at 37°C. The phosphate buffer in the container was continuously exchanged at a rate of 212 ml/h by means of a peristaltic pump. The dialyzed (digested) fraction dripped into a beaker. The dialysate (400 ml) present in the container at the end of the digestion was added to the digested fraction. Thimerosal at a concentration of 50 ppm, was added to all solutions to prevent microbial growth. This did not interfere with digestion and subsequent analysis. Samples of the digested and non-digested (retained in the dialysis membrane) fractions were stored at -20°C for N determination and amino acid analysis. The digestibility of the protein was calculated from the following equation:

$$\% \text{ digestibility} = \frac{\text{N in dialysate}}{\text{N in dialysate + N in dialysis bag}} \times 100$$

The digestibility data (protein and amino acids) were taken from the mean of two experiments.

Amino acid analysis

Samples were hydrolyzed 24 h in 6 N HCl at 100°C under vacuum, evaporated, resuspended in 0.01 N HCl at a concentration of 1 mg protein (N x 6.25)/ml and stored at -20°C. In the samples previously digested enzymatically, the phosphate ions originating from the dialysis buffer had to be removed because they interfered with the derivatization reaction and subsequent chromatography. After acid hydrolysis, the samples were neutralized with N NaOH, $FeCl_3$ added to give a final ratio of Fe to PO_4 of 1.5, centrifuged 15 min at 1500 x *g* and the supernatant processed as above.

The derivatization of amino acids with dansyl chloride was performed according to the method of Schmidt *et al.* (15) modified as follows. A sample of 120 µl acid hydrolysate was placed in vials with 40 nmoles of norleucine as an

internal standard and evaporated under an air stream. The samples were then suspended in 240 µl of 0.5 M Na_2CO_3 pH 10.5 and 600 µl of dansyl chloride (1.25 mg/ml acetone) added. The vials were capped, the contents mixed and heated at 100°C for 4 min. Enzyme digested samples were heated for 10 min to overcome the interferences caused by the remaining phosphate ions, and without affecting the amino acid analysis.

The amino acids were analyzed by high performance liquid chromatography (HPLC) using a liquid chromatograph (Model 5000, Varian, Palo Alto, CA) equipped with an injection valve and loop of 10 µl and an automatic injector. Dansyl amino acids were detected in the U.V. (250 nm) and the recorded peaks integrated automatically. We used a reverse phase column (Model Ultrasphere-ODS, Beckman, Berkeley, CA) of 25 x 0.46 cm with a particle size of 5 µm, 10 µl sample injection and a mobile phase flow rate of 1.2 ml/min. The mobile phase consisted of a mixture of 30 mM phosphate buffer pH 7.5 in acetonitrile varying in the following proportions (% PO_4 in the mixture) with time in minutes: 10% (0), 20% (7), 24% (19), 40% (28), 45% (30), 50% (37), 10% (42), 10% (45).

Using norleucine as an internal standard, the values of all amino acids were calculated in percent on a weight basis and the total equaled 100%. This procedure slightly overestimated the absolute concentration of the amino acids since three of them were not included in the calculations, namely cysteine and tryptophan which were unstable towards acid hydrolysis and histidine which was poorly resolved by chromatography. The amino acid compositions of the proteins were the results of single determinations. Arginine and alanine appear together because they were not separated by chromatography. Amino acid digestibility was calculated as follows: the composition in amino acids of both the digested and the non digested samples was multiplied by the corresponding fraction of N recovered in each. With this calculation, two quantities were obtained for each amino acid, the sum of which equaled the initial protein composition for this amino acid. The equation used for the calculation of protein digestibility was applied to each amino acid.

Statistical analysis

For each of the three proteins, the values of N digestibility were submitted to an analysis of variance and evaluated with Duncan's multiple range test using a computerized system (16).

RESULTS

Lysinoalanine formation was measured in alkali-treated proteins subjected to different conditions of pH, temperature and duration (Figure 1). In general, LAL increased with the severity of the treatment and varied with the nature of the protein. As shown in fig. 1A, lysinoalanine formation increased with pH (measured at 50°C for 4 h); this compound was detectable at pH 11 in the vegetable proteins and only at pH 12 in casein. Under these conditions, rapeseed protein produced most LAL. The effect of temperature was examined at pH 12 for 4 h (fig. 1B). Lysinoalanine was detected at lower temperature (40°C) in casein and rapeseed protein than in soybean protein (50°C). Lysinoalanine increased only slightly thereafter with temperature in the vegetable proteins, in contrast to casein where it increased linearly and reached much higher concentrations. The time-course for the formation of LAL at pH 12 and 50°C is shown in figure 1C. After a steep rise, the process slowed down in all three proteins, but sooner in casein than in the two vegetable proteins. The treatment also affected other amino acids, but to a lesser extent (Tables 1, 2 and 3). In general, a reduction of lysine accompanied LAL formation and was mainly observed after long treatment (more than 4 h) and at high temperature (over 50°C). Treatment of 8 h or at high temperature (80°C) of soybean and

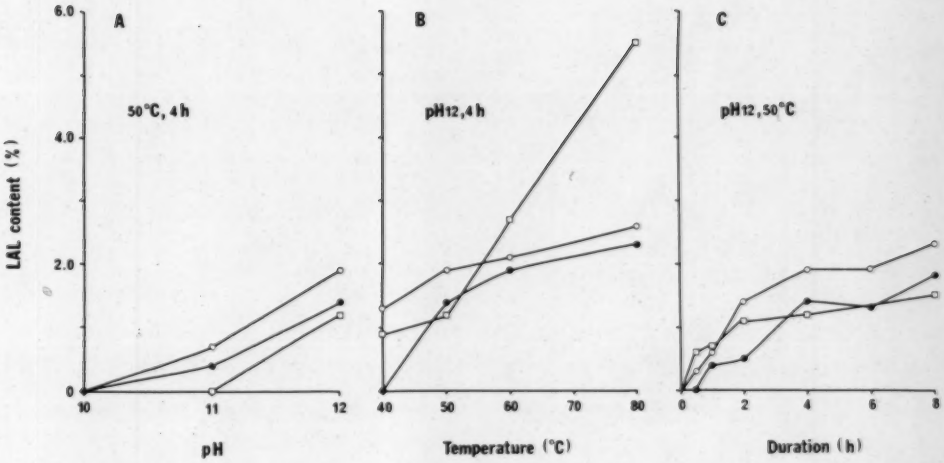

FIG. 1

Effect of Alkaline Treatment on LAL Formation. Soybean Protein, ●; Rapeseed Protein, ○; Casein, □.

TABLE 1

Effect of Alkali Treatment on the Amino Acid Composition (%) of Soybean Protein

Treatment:												
pH		10	11	12	12	12	12	12	12	12	12	12
Temp., °C	Control	50	50	40	50	60	80	50	50	50	50	50
Duration, h		4	4	4	4	4	4	0.5	1	2	6	8
ASP	9.6	8.4[1]	10.6	10.9	9.2	10.3	10.5	9.9	8.7	11.1	9.4	9.4
GLU	15.7	13.9	17.3	17.1	14.6	16.2	14.0	15.2	15.8	18.4	14.9	14.6
SER	4.0	4.1	4.1	3.7	4.8	4.5	5.0	3.9	4.8	3.7	4.4	4.1
THR	1.8	1.5	1.6	1.6	1.7	1.8	1.7	1.7	2.1	1.4	1.6	0.8
GLY	5.8	6.3	5.4	6.1	5.7	5.6	6.5	6.5	5.6	6.1	6.2	6.3
ARG + ALA	16.3	15.8	15.4	15.0	14.9	15.3	16.5	16.0	16.0	15.9	16.9	17.0
PRO	5.6	5.6	5.3	5.6	5.6	6.0	5.7	5.8	5.5	5.2	5.4	6.0
VAL	4.7	5.0	4.7	4.6	4.7	4.5	4.8	4.9	5.0	4.7	4.4	5.0
MET	0.0	0.0	0.0	0.0	0.6	0.2	0.0	0.0	1.1	0.0	0.4	0.0
ILE	5.3	5.6	5.1	6.2	6.3	5.3	6.2	6.2	5.1	5.9	5.8	5.4
LEU	11.1	11.6	11.2	10.2	10.7	11.3	11.3	11.0	12.0	10.8	10.4	12.2
PHE	7.2	9.1	7.3	5.7	8.5	6.7	4.7	6.3	7.6	5.4	6.0	6.0
LAL	0.0	0.0	0.4	0.0	1.4	1.9	2.3	0.0	0.4	0.5	1.3	1.8
LYS	6.5	6.9	6.3	6.0	6.0	5.9	5.9	6.7	7.1	7.3	5.7	5.9
TYR	5.7	5.2	5.4	5.9	4.4	4.6	5.2	5.9	4.5	3.4	5.6	5.5

[1] Analyses of alkali-treated products refer to proteins separated from the aqueous suspension by acid precipitation after alkali treatment as described in Materials and Methods.

TABLE 2

Effect of Alkali Treatment on the Amino Acid Composition (%) of Rapeseed Protein

Treatment:												
pH		10	11	12	12	12	12	12	12	12	12	12
Temp., °C	Control	50	50	40	50	60	80	50	50	50	50	50
Duration, h		4	4	4	4	4	4	0.5	1	2	6	8
ASP	6.3	7.9[1]	8.3	8.6	8.8	7.2	9.2	8.1	7.1	8.3	7.5	8.5
GLU	18.6	17.9	20.7	19.1	17.7	16.2	19.5	15.4	15.5	18.5	14.7	17.5
SER	3.2	4.8	4.1	5.1	5.1	4.5	5.5	4.2	5.1	5.5	4.4	5.0
THR	2.2	1.7	1.6	1.9	1.7	2.1	2.0	1.4	1.9	1.9	2.1	2.4
GLY	7.2	6.9	6.8	6.8	6.8	7.3	7.1	7.7	7.4	6.6	7.2	6.9
ARG + ALA	16.6	16.3	15.3	16.1	16.2	16.1	16.3	17.2	18.4	17.4	17.7	16.3
PRO	7.0	6.5	5.7	5.8	6.2	6.2	5.6	6.3	6.0	5.3	5.9	4.4
VAL	5.2	5.1	4.6	4.9	4.7	4.9	4.7	5.2	5.2	4.6	4.9	4.4
MET	1.4	0.9	1.4	0.5	1.6	1.5	1.2	1.4	1.5	0.7	1.4	2.0
ILE	5.7	5.8	4.9	5.3	5.3	5.3	5.3	5.5	5.2	5.1	5.2	5.8
LEU	10.9	10.1	10.0	10.1	9.9	10.7	9.7	11.2	10.6	9.6	10.5	11.0
PHE	5.6	6.1	6.0	5.5	4.9	5.5	4.6	6.0	5.3	5.9	6.0	5.6
LAL	0.0	0.0	0.7	1.3	1.9	2.1	2.4	0.3	0.6	1.4	1.9	2.3
LYS	6.2	6.2	5.6	5.3	5.1	5.0	4.4	5.7	5.9	5.4	5.1	4.7
TYR	3.9	3.7	4.3	3.7	4.1	5.4	2.4	4.7	4.4	3.7	5.3	3.4

[1] Analyses of alkali-treated products refer to proteins separated from the aqueous suspension by acid precipitation after alkali treatment as described in Materials and methods.

TABLE 3

Effect of Alkali Treatment on the Amino Acid Composition (%) of Casein

Treatment:												
pH		10	11	12	12	12	12	12	12	12	12	12
Temp., °C	Control	50	50	40	50	60	80	50	50	50	50	50
Duration, h		4	4	4	4	4	4	0.5	1	2	6	8
ASP	6.2	6.0[1]	5.8	6.1	7.2	6.8	5.6	5.8	5.5	7.2	5.8	7.5
GLU	19.1	18.5	18.0	19.5	20.6	19.4	17.6	17.4	18.0	20.4	18.5	20.9
SER	5.4	6.3	5.1	5.7	6.2	4.8	4.8	5.3	6.0	5.6	5.4	5.7
THR	2.9	3.6	2.1	2.1	2.7	0.7	1.2	2.3	2.1	1.4	1.7	2.5
GLY	2.4	2.4	2.9	2.8	2.3	2.2	2.5	2.0	2.6	2.4	2.5	2.4
ARG + ALA	10.0	10.6	10.8	9.8	9.1	9.3	9.7	10.2	10.0	10.6	10.1	9.3
PRO	10.7	10.0	10.7	10.8	10.3	10.1	10.5	10.8	10.9	9.7	10.0	10.0
VAL	5.5	5.1	5.6	5.7	5.5	5.2	5.5	5.6	5.7	5.2	5.2	5.3
MET	1.8	1.8	1.5	1.2	1.1	1.4	1.9	2.4	1.6	1.8	2.0	1.1
ILE	6.1	5.5	5.5	6.1	5.8	5.7	6.1	5.0	6.0	5.8	5.6	5.9
LEU	10.9	10.5	11.4	11.4	10.7	12.4	11.4	12.2	11.2	10.4	10.4	10.4
PHE	5.4	5.2	5.3	5.1	4.8	7.1	5.9	6.3	5.7	5.7	5.4	5.4
LAL	0.0	0.0	0.0	0.9	1.2	2.7	5.5	0.6	0.7	1.1	-	1.5
LYS	7.8	8.5	8.1	7.0	7.4	6.3	5.8	7.8	8.1	7.2	7.1	6.6
TYR	5.8	6.1	7.2	5.8	5.6	6.0	6.1	6.4	6.1	5.7	5.6	5.6

[1] Analyses of alkali-treated products refer to proteins separated from the aqueous suspension by acid precipitation after alkali treatment as described in Materials and methods.

rapeseed proteins reduced proline and phenylalanine content. In soybean protein (Table 1) a reduction of threonine was also noted after 8 h of treatment. In casein (Table 3) threonine and serine were also decreased at 60°C and 80°C, and this reduction was accompanied by a high production of lysinoalanine.

Each protein source was submitted to a two-stage in vitro digestion with continuous removal of digested products by dialysis. This allowed the measurement not only of the effect of alkali treatment on total protein digestibility, but also the rate of release of each amino acid. Table 4 presents the digestibility of the whole protein on the basis of dialysed nitrogen. In a 24-hour digestion, casein and soybean protein were digested to the same extent and up to 85%, which was 10% more than rapeseed protein. At 50°C, increasing pH of treatment did not affect total casein and rapeseed protein digestibility, but pH 10 markedly reduced digestion of soybean protein, which returned to normal at higher pH. At pH 12, duration of alkali treatment did not significantly affect the digestibility of the three proteins except after a 2-h treatment of casein ($P<0.05$). On the other hand, raising the temperature of treatment over 50°C reduced protein digestibility in the following decreasing order: casein > soybean protein > rapeseed protein.

The influence of the protein and alkaline treatment on amino acid release was evaluated for a given treated protein sample by comparing the amino acids to the whole protein (N) on the basis of their relative digestibility. The amino acid digestibility of a given protein or treated protein sample was expressed as the ratio of the digestibility of each amino acid to that of the whole protein (Tables 5, 6 and 7). The degree of release of amino acids was not related to their concentration in the protein but it was influenced by the nature of the protein. There was much less deviation in the rate of amino acid release from rapeseed protein than from the two other proteins which resembled each other in this aspect. Using data from untreated proteins, amino acids were classified on the basis of their relative rate of liberation (Table 8). Since the data were inconsistent for methionine these values were not included. Digestive enzymes released aromatic and basic amino acids faster than the dicarboxylic amino acids, whereas neutral amino acids could not be classified from their rate of release. Most essential amino acids were liberated rapidly in all three proteins. This would also include methionine (casein and rapeseed protein only). Isoleucine was slowly liberated in all three proteins, also valine in casein and rapeseed protein. Proline was very resistant to digestion in the vegetable proteins and in contrast to casein where this amino acid was well released. Conversely, serine was very resistant to digestion in casein compared to its release in the vegetable proteins.

Tables 5, 6 and 7 show also the effect of heat, alkali and duration of treatment on the relative digestibility of each amino acid. In the vegetable proteins, lysinoalanine was liberated much less than the whole protein. Even with incomplete results due to the difficulty of accurately measuring LAL in proteins containing little LAL, it can be seen that in the vegetable proteins, the LAL digestibility was not correlated with the severity of treatment, LAL content or protein digestibility. On the other hand, LAL was more readily released from casein, after high temperature treatment, than in other casein samples containing less LAL.

The relative rate of hydrolytic release of other amino acids varied with the nature of the protein as well as the type of treatment. In soybean protein (Table 5), treatment at pH 10 for 4 h, accelerated the liberation of some rapidly released amino acids (tyrosine, threonine, arginine + alanine), but the slowly released amino acids (glutamic acid, aspartic acid, proline) as well as glycine and lysine were less readily liberated under these conditions. This effect was abolished when the treatment pH was raised. At pH 12, a similar distribution was observed on raising the treatment temperature to 60°C resulting

TABLE 4

Effect of Alkaline Treatment on Protein Digestibility in %

Treatment:												
pH		10	11	12	12	12	12	12	12	12	12	12
Temp., °C	Control	50	50	40	50	60	80	50	50	50	50	50
Duration, h		4	4	4	4	4	4	0.5	1	2	6	8
SOYBEAN	84	69	77	75	77	65	61	76	81	72	78	76
	a[1]	bcd	abc	abc	abc	cd	d	abc	ab	abcd	abc	abc
	A	AB	AB	AB	AB	AB	B	AB	A	AB	AB	AB
RAPESEED	74	78	75	79	66	62	62	76	73	74	68	75
	ab	a	ab	a	ab	b	b	ab	ab	ab	ab	ab
	A	A	A	A	A	A	A	A	A	A	A	A
CASEIN	86	83	82	76	82	64	52	81	76	72	76	79
	a	ab	ab	abc	ab	cd	d	ab	abc	bc	abc	ab
	A	AB	AB	AB	AB	BC	C	AB	AB	AB	AB	AB

[1] Values not accompanied by the same letter are significantly different: lower case letters, $P < 0.05$; capital letters, $P < 0.01$.

TABLE 5

Effect of Alkaline Treatment on the Relative Amino Acid Digestibility of Soybean Protein (Whole Protein Digestibility = 1)

Treatment:												
pH		10	11	12	12	12	12	12	12	12	12	12
Temp., °C	Control	50	50	40	50	60	80	50	50	50	50	50
Duration, h		4	4	4	4	4	4	0.5	1	2	6	8
ASP	0.92	0.80	0.84	0.83	0.88	0.82	0.70	0.91	0.80	0.94	0.94	0.98
GLU	0.86	0.71	0.85	0.80	0.90	0.84	0.72	0.85	1.00	0.91	0.92	0.86
SER	1.04	1.08	1.04	1.04	0.99	0.95	1.00	1.04	1.03	0.97	1.00	0.99
THR	1.08	1.19	1.04	1.13	1.01	1.05	1.17	1.08	1.05	1.03	1.04	0.98
GLY	1.01	0.78	0.96	0.89	0.94	0.84	0.89	0.88	0.90	0.82	0.83	0.99
ARG + ALA	1.02	1.19	1.00	1.09	1.02	1.07	1.17	1.07	1.05	1.01	1.04	1.01
PRO	0.92	0.82	0.86	0.82	0.86	0.77	0.79	0.91	0.85	0.80	0.89	0.83
VAL	1.04	1.06	1.05	1.05	1.05	1.00	1.02	1.02	1.02	0.98	1.00	0.99
MET	ND[1]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ILE	0.99	1.01	0.97	0.96	0.98	0.91	0.92	0.95	1.04	0.96	0.90	0.91
LEU	1.03	1.07	1.04	1.08	1.05	1.11	1.13	1.07	1.04	1.05	1.07	1.05
PHE	1.11	1.15	1.12	1.15	1.11	1.16	1.24	1.08	1.04	1.16	1.07	1.09
LAL	-	-	NM[2]	-	NM	0.54	0.60	-	0.41	1.05	NM	0.80
LYS	1.01	0.92	0.99	1.04	1.03	1.14	1.19	0.8	1.01	1.02	1.04	1.04
TYR	1.11	1.19	1.04	1.09	1.11	1.20	1.11	1.11	0.84	1.17	1.10	1.09

[1] ND: not determined.
[2] NM: not measurable.

TABLE 6

Effect of Alkaline Treatment on the Relative Amino Acid Digestibility of Rapeseed Protein (Whole Protein Digestibility = 1)

Treatment:												
pH		10	11	12	12	12	12	12	12	12	12	12
Temp., °C	Control	50	50	40	50	60	80	50	50	50	50	50
Duration, h		4	4	4	4	4	4	0.5	1	2	6	8
ASP	0.97	0.97	0.91	0.90	0.87	0.81	1.01	0.91	0.94	1.02	0.99	0.96
GLU	0.94	0.95	0.93	0.94	0.93	0.80	1.01	0.95	0.91	0.96	0.97	0.93
SER	1.02	1.01	1.03	1.02	1.07	1.07	0.92	1.03	1.06	1.04	1.08	1.05
THR	1.05	1.05	0.97	1.09	1.21	1.11	0.77	1.06	1.07	1.03	1.21	1.04
GLY	0.90	0.95	0.93	0.94	0.91	0.91	0.95	0.88	0.93	0.93	0.93	0.94
ARG + ALA	1.04	0.99	1.05	1.03	1.06	1.08	0.98	1.03	0.98	0.99	1.06	1.03
PRO	0.86	0.90	0.86	0.91	0.80	0.91	0.91	0.88	0.92	0.92	0.92	0.88
VAL	0.91	0.97	0.97	0.97	0.92	0.98	0.98	0.95	0.96	0.97	0.99	0.96
MET	0.88	1.19	1.12	1.01	0.00	1.29	0.79	1.19	1.19	0.72	1.38	0.45
ILE	0.88	0.98	0.98	0.98	0.96	0.96	0.88	0.95	0.95	0.94	0.93	0.95
LEU	0.97	1.01	1.01	0.99	1.02	1.01	1.01	1.00	0.99	0.98	1.03	1.00
PHE	0.92	1.03	1.05	1.02	1.09	1.06	0.98	1.07	1.07	1.00	1.04	1.07
LAL	-	-	0.68	NM[1]	0.22	0.34	0.30	0	0	0.54	0.74	0.23
LYS	1.06	1.05	1.05	1.03	1.06	1.06	1.02	1.03	1.05	0.98	1.05	1.04
TYR	1.06	1.04	0.94	1.10	1.14	1.14	1.10	1.08	1.08	1.02	0.90	1.10

[1] NM: not measurable.

TABLE 7

Effect of Alkaline Treatment on the Relative Amino Acid Digestibility of Casein (Whole Protein Digestibility = 1)

Treatment:												
pH		10	11	12	12	12	12	12	12	12	12	12
Temp., °C	Control	50	50	40	50	60	80	50	50	50	50	50
Duration, h		4	4	4	4	4	4	0.5	1	2	6	8
ASP	0.97	0.98	1.05	0.99	0.97	0.64	0.97	0.93	0.84	0.88	0.80	0.88
GLU	0.94	0.96	0.98	0.92	0.93	0.72	1.00	0.89	0.78	0.86	0.77	0.89
SER	0.88	0.88	0.84	0.84	0.84	0.90	0.97	0.84	0.81	0.81	0.83	0.84
THR	1.02	1.04	1.04	1.06	1.00	1.13	1.42	1.06	1.01	1.00	0.91	0.99
GLY	0.96	0.94	0.91	0.88	0.93	0.91	0.97	0.94	0.98	0.93	0.97	0.94
ARG + ALA	1.03	1.03	0.99	1.02	1.01	1.11	0.84	1.04	1.02	1.03	1.06	1.05
PRO	1.01	0.99	0.96	0.92	0.97	0.94	1.04	0.98	0.96	0.95	0.98	0.97
VAL	0.96	0.94	0.90	0.90	0.98	0.90	0.99	0.95	0.94	0.92	0.97	0.94
MET	1.07	0.70	0.93	0.57	1.15	0.98	0.89	1.11	1.21	1.29	1.18	1.18
ILE	0.88	0.87	0.82	0.81	0.82	0.83	0.96	0.87	0.87	0.80	0.86	0.87
LEU	1.07	1.06	1.05	1.10	1.06	1.11	0.94	1.05	1.09	1.08	1.11	1.08
PHE	1.07	1.08	1.09	1.07	1.07	1.17	1.00	1.09	1.10	1.12	1.09	1.10
LAL	-	-	-	0.60	0.26	1.00	1.17	NM[1]	NM	NM	-	0.44
LYS	1.11	1.12	1.11	1.15	1.12	1.22	0.96	1.14	1.17	1.16	1.17	1.12
TYR	1.12	1.14	1.14	1.16	1.12	1.35	0.92	1.14	1.20	1.23	1.22	1.16

[1] NM: not measurable.

TABLE 8

Chart of Amino Acids in Decreasing Order of Release Relatively to the Protein (N)[1]

Soybean	TYR PHE THR SER VAL LEU ARG + ALA GLY LYS $\overline{N}$ ILE ASP PRO GLU
Rapeseed	TYR LYS THR ARG + ALA SER $\overline{N}$ LEU ASP GLU PHE VAL GLY ILE PRO
Casein	TYR LYS PHE LEU ARG + ALA THR PRO $\overline{N}$ ASP GLY VAL GLU ILE SER

[1] Methionine was excluded for lack of repeatability in its determination.

in a gradual reduction in the digestibility of aspartic acid, glutamic acid, proline and glycine and improvement of the release of basic and aromatic amino acids. This specific temperature effect disappeared at 80°C. Duration effects were measured at pH 12 and 50°C. Under these conditions, the liberation of tyrosine and lysine was severely reduced at the beginning of the treatment and returned to normal thereafter.

With rapeseed protein (Table 6), temperature at pH 12 affected the rate of release of amino acids between 50 and 60°C, in the same fashion as for soybean protein, increasing the digestibility of threonine and decreasing that of proline, glutamic acid and aspartic acid. The duration of treatment also affected the release of different amino acids but in a less organized fashion, with a peak after 4 h.

Amino acid release from casein (Table 7) was much less affected than in the vegetable proteins by alkali treatment. The relative digestibility of its constituent amino acids did not vary much with pH and duration of treatment. On the other hand, heat treatment at 60°C markedly modified the process of digestion, leading to increased liberation of the rapidly released amino acids, i.e. tyrosine, lysine and phenylalanine, and reduction in the liberation of slowly released amino acids, i.e. aspastic acid and glutamic acid. These changes were reversible and disappeared at 80°C.

DISCUSSION

Lysinoalanine is found in a number of food proteins heat-treated at neutral pH although its formation occurs mainly under alkaline conditions (6,11,17). In our experiments, LAL was detected at pH 11 with more extensive formation at pH 12. Its formation occurs at the expense of its precursor amino acids. The apparent non-quantitative relationship we obtained comes from the fact that results are presented in percent on a weight basis: for each percent LAL formed, lysine should decrease by only 0.63% and serine by only 0.45%. On the other hand, the ε-amino group of lysine would preferentially react with the dehydroalanine originating from the more easily alkali-transformed cystine (6) not measured in this experiment. However, the cystine content of casein (0.4%) could not account for all the dehydroalanine needed for LAL formation. Some dehydroalanine must come from another source as serine, the content of which was reduced only at temperatures above 50°C.

The process of LAL formation was linear for only a few hours in all three proteins and then slowed down even when the amino acid precursors in the protein were far from being exhausted. Friedman (6) explained this phenomenon and proposed that the specific structure of the protein played an important role. The fusion of the two precursor residues would only occur if both were situated on the same or closely adjacent protein chains. Therefore, after these convenient sites have been used up, additional LAL forms less readily. The process would be hindered by the protein rigidity as well as by the newly formed LAL crosslinks.

In contrast to LAL formation in vegetable proteins, LAL formation in casein increased linearly with temperature up to 80°C as reported elsewhere (8). The low content of disulfide bonds in casein and its high proline content makes it a non rigid protein and consequently highly deformable as for example by heat (18). Thus, LAL precursors in this protein would be easily brought together on heating to favour LAL formation. This is in contrast to the situation in soybean proteins where the structure is more rigid (19).

The treated proteins were digested enzymatically in vitro to achieve a better understanding of the nutritional effect of alkali treatment. The principal feature of our digestion method consisted in the simultaneous and continuous dialysis of digestion products to prevent inhibition of proteolysis. Dialysis simulated absorption by the intestine where digestion products were prevented from accumulating (20). This was probably reflected in the amino acid digestibility values. As shown in tables 6, 7 and 8, there was only a small variation in the rate of liberation of the individual amino acids. This resembles in vivo amino acid digestibility values where the difference between the least and the most rapidly digested amino acid remained around 15% (21) and in contrast to in vitro digestion performed in a closed reaction vessel (3). To explain the differences between the two situations, it is suggested that the delayed liberation of the slowly released amino acids could not be corrected by time since enzyme activity is stopped by the accumulating digestion products before digestion is completed. Dialysis would relieve this specific inhibition which yields a biased in vitro evaluation of amino acid availability.

The observations about amino acid digestibility apply also but to a lesser extent to the work of Stahmann and Woldegiorgis (2) where essential amino acids were released on the average three times faster than non essential amino acids, and to the study of Mauron (1) where they were released four times faster with a system using dialysis. In the latter, dialysis might not be sufficient to readily remove digestion products. On the other hand, in the above mentioned studies, digested material included only amino acids released free, whereas in our experiments, we included also amino acids liberated as very short peptides. This could explain the differences with these studies since certain amino acids might be more readily released in the form of short peptides (22). This is the form in which the proteins are preferentially absorbed in vivo instead of free amino acids (22, 23). Consequently, the measurement of only free amino acids released to evaluate digestion is questionable. The relevance to the in vivo situation of both approaches needs to be evaluated properly.

The order of release of amino acids relative to the protein is presented in table 8. Basic and aromatic amino acids, and leucine were released faster than the rest of the protein in all three proteins. This probably reflects the specificity of the digestive enzymes for these amino acids (24, 25). This series of rapidly released amino acids includes most essential amino acids to which should be added threonine as well as methionine; the latter was not included in the calculations but it was also released rapidly. In contrast, two essential amino acids were released slowly: isoleucine, in all three proteins and valine in casein and rapeseed protein.

Differences in the availability of amino acids would also arise from the nature of the protein due to its structure and amino acid sequence. For example, the amino acid release in rapeseed was more uniform than is casein and soybean protein. Leucine was more available in the former protein. Finally, the vegetable proteins differed from casein by a reduced proline digestibility and by a higher serine digestibility.

Heat treatment reduced the in vitro digestibility of the proteins as reported elsewhere (2,4,7). Casein was more heat labile than the vegetable proteins. This protein possesses an open porous structure into which large molecules such as enzymes can diffuse (26). Upon heating, it is highly deformable and aggregates into clusters (18,28), so that enzyme access to the protein would be reduced. Although they aggregate upon heating (28), soybean proteins possess more rigid structures (19) and would resist the denaturing effect of heat better than casein. In addition, a reduced digestibility of the proteins could be brought about by chemical transformations of amino acids by alkali. These are: 1- the disappearance of lysine residues, a preferred target of digestive enzymes such as trypsin and carboxypeptidase B; 2- the formation of LAL crosslinks which would hinder enzyme action; 3- the racemization of amino acids by alkali. For instance, the number of peptide bonds hydrolyzed by digestive enzymes is inversely related to racemization since the proteolytic enzymes function better with L-L peptide bonds (7,8,29).

Heat and alkali treatment produced other more specific changes in protein digestion. At pH 12, temperatures between 50 and 60°C induced reversible alterations in all three proteins as shown by modifications in the digestibility of slowly and rapidly released amino acids (tables 5, 6 and 7). This phenomenon should correspond to a turning point for the stability of the protein structure with consequent changes in enzyme attack. These transient modifications were more pronounced in soybean protein where they occurred also at pH 10. Thus, from a nutritional viewpoint, it could be more relevant to examine treatment effect on the digestibility of limiting amino acids rather than on protein digestibility.

The results of LAL digestibility were not consistent in protein samples containing little of this amino acid, but it was generally the least digested. This helps to explain its low availability in vivo and its weak toxic potential (10,12,14). On the other hand, LAL was as well digested as the protein in severely treated casein samples at 60 or 80°C. This indicates that LAL availability could vary with the source of protein and the type of treatment applied, so that conclusions drawn from one situation may not be generalized. On the other hand, results of LAL digestibility in casein samples containing high amounts of this amino acid are at variance with other reports where an inverse relationship between LAL digestibility and its content in the protein has been reported (12,14). However, the conclusions drawn with lactalbumin (14) may not hold for casein. In the other study (12) excessively low LAL digestibility was reported, resulting from the in vitro digestion procedure used (in a closed reaction vessel). As already mentioned above, care should be taken when interpreting data from such procedures.

With our method, it was possible to detect not only gross changes in the digestion of treated proteins but also more discrete alterations at the level of amino acid digestibility. With complementary measurement of the extent of amino acid racemization, the method should be considered as a suitable tool for protein quality evaluation and a screening test to replace animal assays. With the continuous dialysis, selection of digestion products comparable to those present in in vivo digestion is made possible. The ultimate validity of the method remains to be confirmed by biological assays.

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SERUM AND SECRETORY PROTEINS IN IRON-DEFICIENT RAT PUPS AND DAMS

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ABSTRACT

The effects of iron status during reproduction on serum and secretory proteins in rat dams and pups were studied. Pregnant rats were fed ad libitum diets containing 10 or 250 ppm iron throughout gestation and lactation. Litters were adjusted on day 1 to contain 7 pups, and on day 17, dams and pups were sacrificed. Iron status was determined, and concentrations of various serum and secretory proteins were measured. Iron-deficient pups had lower hemoglobin, serum iron, and liver iron compared to controls. Serum albumin and globulin concentrations were significantly increased in iron-deficient pups. Pup serum lysozyme and myeloperoxidase levels were unchanged by iron deficiency. The serum protein profile of dams was relatively unaltered by dietary iron deficiency. Milk iron concentration was significantly decreased in iron-deficient dams; however, milk from all dams was similar in concentration of three immunoproteins measured: lysozyme, peroxidase, secretory IgA. Salivary total protein, lysozyme, and secretory IgA concentrations were similar between groups of dams. It is concluded that dietary iron deficiency during reproduction which does not retard growth of pups but does deplete iron stores has a minimal effect on the secretory immunoproteins measured.

Key Words: Anemia, hypochromic; Reproduction; Iron; Immunity, natural; Lysozyme; Peroxidases

INTRODUCTION

The effects of iron status on immune response are of interest, because dietary iron deficiency is a common nutritional problem among infants and pregnant women, and because iron deficiency has been reported to both enhance and inhibit infections. Human subjects and animal models have been used to study the effects of dietary iron deficiency on immunity. The majority of investigators have reported that the cell-mediated and humoral components of the immune system are impaired by iron deficiency (1).

The purpose of our experiment was to examine another component of the immune system--secretory immunity--which includes host defense mechanisms present in body fluids, such as serum, saliva, mucus, and milk. There is little information on the effects of iron status on secretory immunity, with the exception of the enzyme lysozyme (EC 3.2.1.17). Lysozyme is released from granules of leukocytes and lyses bacteria by its action on cell-wall mucopolysaccharides. Sherman and Wolinsky have found that iron deficiency during reproduction (2) and growth (3) in rats alters serum and/or tissue lysozyme patterns.

In view of the scant information on iron status and secretory immunity, we investigated the effects of dietary iron deficiency during reproduction on various serum and secretory proteins. Two serum proteins secreted by leukocytes and monocytes, lysozyme and peroxidase (EC 1.11.1.7), in addition to proteins in milk and saliva, were measured at the end of lactation. Our rat model of iron deficiency during reproduction produced anemia in pups without retarding body growth.

MATERIALS AND METHODS

Nulliparous, Sprague-Dawley female rats (Harlan Industries, IN) weighing 180-200 g were housed in individual, stainless-steel cages in animal rooms with controlled temperature (21-22°) and lighting (12 h light-dark cycle). When their weights reached 200-220 g, rats were mated and pregnancy confirmed by the presence of sperm in vaginal smears. Pregnant rats were randomly assigned to an experimental group and fed the appropriate diet throughout gestation and lactation. Dams were placed in solid-bottom maternity cages with iron-free bedding prior to delivery of their pups.

The basal diet, made without addition of iron, contained 5 ppm iron (Table 1). The experimental diets were made by supplementing the basal diet with ferrous sulfate ($FeSO_4 \cdot 7H_2O$) to obtain iron concentrations of 10 and 250 ppm. All diets were supplemented with cupric carbonate at the expense of sucrose to obtain a copper concentration of approximately 12 ppm, since Cerklewski (4) has reported that 9 ppm copper is necessary for optimal copper status in rats during reproduction and in the offspring at weaning. Dietary iron and copper concentrations were measured by atomic absorption spectrophotometry.

TABLE 1

Basal Diet Composition

	Percent
Casein[1]	22
Sucrose	30
Cornstarch	31.5
Corn Oil[2]	10
Cellulose[3]	2
Vitamin Mix[4]	1
Iron-free AIN-76 mineral mix[5]	3.5

[1]Vitamin-free test casein, Teklad, Madison, Wisconsin.

[2]Mrs. Tucker's Pure Corn Oil, Anderson Clayton Foods, Dallas, Texas.

[3]Alphacel, ICN Nutritional Biochemical Co., Cleveland, Ohio.

[4]#40060, Teklad, Madison, Wisconsin: Composition of Vitamin Mix (g/kg vitamin mix): P-aminobenzoic acid, 11.0132; Ascorbic acid, coated (97.5%), 101.6604; Biotin, 0.0441; Vitamin B_{12}, 2.9736; Calcium pantothenate, 6.6079; Choline dihydrogen citrate, 349.6916; Folic acid, 0.1982; Inositol, 11.0132; Menadione (Vitamin K_3), 4.9559; Niacin, 9.9119; Pyridoxine HCl, 2.2026; Riboflavin, 2.2026; Thiamin HCl, 2.2026; Dry Vitamin A palmitate (500,000 U/g), 3.9648; Dry Vitamin D_2 (500,000 U/g), 0.4405; Dry Vitamin E acetate (500 U/g), 24.2291; Cornstarch, 466.6878.

[5]Teklad, Madison, Wisconsin: Composition of Mineral Mix (g/kg mineral mix): $CaHPO_4$, dibasic, 500.0; NaCl, 74.0; Potassium citrate, monohydrate, 220.0; K_2SO_4, 52.0; MgO, 24.0; $FeSO_4 \cdot 7H_2O$, 6.0; $ZnCO_3$, 1.6; $MnCO_3$, 3.5; Cupric Carbonate, 0.3; KIO_3, 0.01; $Na_2SeO_3 5H_2O$, 0.01; $CrK(SO_4)_2 \cdot 12H_2O$, 0.55; Sucrose, 118.03.

Two groups of pregnant rats (n=12 rats/group) were fed diets containing one of two iron levels, 10 or 250 ppm, throughout gestation and lactation. Diets and demineralized water were provided ad libitum. The 10 ppm iron diet was chosen to produce iron deficiency in which iron stores and hemoglobin levels were decreased in pups, but body weights were normal. A dietary iron level of 250 ppm has been determined by our laboratory to produce optimal iron status in dams and pups during gestation and lactation (5).

Litters were adjusted on day 1 to contain 7 pups. On day 17 of lactation, dams and their pups were separated for 4 hours before sacrificing to allow accumulation of milk. Dams were anesthetized with sodium pentobarbital (0.04 ml of 60 mg/ml solution per 100 grams body weight) and injected intraperitoneally with oxytocin (0.025 ml of 10 USP units/ml per 100 grams body weight) to facilitate milking and with pilocarpine to stimulate salivation (0.2 ml of 3 mg/ml solution per 100 grams body weight). Milk and

saliva were collected using a suction apparatus in which the fluids flowed into capillary tubing attached to a test tube kept in an ice bath.

Pups were anesthetized with chloroform vapors. Blood was collected from all animals by cardiac puncture, allowed to clot, and centrifuged (Centra-7, International Equipment Company, Needham Heights, MA) at 2800 rpm for 10 minutes. Sera from pups of each litter were pooled and all sera were frozen at -20°. Liver, spleen and thymus were removed from each pup, weighed and frozen. All equipment used in diet preparation, animal feeding, and sacrificing was stainless-steel or acid-washed glass to avoid iron contamination.

Tail-blood samples were collected for determination of hemoglobin concentrations, microhematocrits (6) and peripheral white blood cell counts (Coulter Counter Model F, Hialeah, FL). Three livers from each litter were pooled for iron analysis. Livers were dry-ashed at 550° for 24 h (Blue M Muffle Furnace, Scientific Products, McGaw Park, IL) and iron concentrations determined by atomic absorption spectrophotometry (Jarell-Ash, Fisher Scientific Co., Pittsburgh, PA). Serum iron concentrations were determined according to the method of Brittenham (7). Transferrin concentration was calculated from total iron-binding capacity (8).

Total protein concentration was determined by the method of Lowry et al. (9) using lysozyme (Sigma Chemical Co., St. Louis, MO) as the standard. Albumin and globulin fractions were measured using colorimetric methods (10,11). Immunoglobulins G, M and A were quantitated by radial immunodiffusion (12) using rat standards (Miles Biochemicals, Elkhart, IN). Lysozyme concentration was measured on lysoplates (13) and peroxidase determined using 4-aminoantipyrine as the hydrogen donor (14) and horseradish peroxidase (Sigma) as the standard.

Milk samples were centrifuged at 500 rpm for 15 minutes, and the whey fraction used in all analyses. Saliva samples were centrifuged (Sorvall Superspeed RC2-B, Newtown, CT) at 8200 rpm for 10 minutes, the precipitate discarded, and remaining supernate used for analyses.

Statistical significance was determined between the two group means by Student's t-test.

RESULTS AND DISCUSSION

Initial and final body weights of the two groups of dams did not differ. Food intakes were similar for all rats during gestation and lactation (data not shown).

The body weights of iron-deficient 17-day-old rat pups were slightly lower than controls, although this difference was not statistically significant (Table 2). Hemoglobin, serum iron, and liver iron concentrations were decreased in iron-deficient pups. Iron-deficient pups had smaller thymuses and spleens on both absolute and body weight bases than controls. This confirms previous work by Rothenbacher and Sherman (15) and Kochanowski and Sherman (16) documenting changes in structure and cellularity in key lymphoid tissues associated with iron deficiency.

TABLE 2

Body and Organ Weights and Iron Status
of 17-day-old Rat Pups

	10 ppm Fe	250 ppm Fe
Body Weight, g	32.2 ± 1.3[1]	35.4 ± 0.8
Thymus Weight, g	0.110 ± 0.007	0.202 ± 0.011*
Spleen Weight, g	0.112 ± 0.005	0.148 ± 0.008*
Hemoglobin, g/dl	5.1 ± 0.3	8.7 ± 0.3*
Serum Iron, µg/dl	39.8 ± 2.9	102.9 ± 7.6*
Liver Iron, µg/g	75 ± 5	162 ± 8*

[1]Values expressed as mean ± SEM, n=9-12 litters/group.

*Indicates statistical significance $p < 0.005$.

Serum protein fractions have not been previously measured in iron-deficient rat pups. Total serum protein concentration was significantly increased in iron-deficient pups (Table 3). This increase was observed in both the globulin and albumin fractions. The increased globulin concentration was not due to increased immunoglobulin (Ig) concentrations. In fact, serum IgG, IgM and IgA were all slightly decreased in iron-deficient pups compared to controls. Most likely, the increased globulin is due to transferrin, a β-1 globulin which was significantly increased ($p < 0.001$) in iron-deficient pups (425 ± 10 mg/dl) compared to controls (310 ± 17 mg/dl). The rise in serum albumin in iron-deficiency is probably due to hemoconcentration or dehydration (17) rather than increased synthesis, since protein biosynthesis has been reported to be unchanged or decreased (18,19) in iron deficiency.

TABLE 3

Serum Proteins--17-day-old Rat Pups

	10 ppm Fe	250 ppm Fe
Total Protein, g/dl	4.56 ± 0.08[1]	4.08 ± 0.08*
Albumin, g/dl	3.15 ± 0.04	2.91 ± 0.05*
Globulin, g/dl	1.57 ± 0.03	1.40 ± 0.03*
IgG, mg/dl	539 ± 42	673 ± 78
IgM, mg/dl	23 ± 3	27 ± 3
IgA, mg/dl	29 ± 4	38 ± 4
Lysozyme, µg/g protein	20.1 ± 2.6	23.4 ± 2.6
Peroxidase,[2] mU/mg protein	0.52 ± 0.10	0.55 ± 0.10

[1]Values expressed as mean ± SEM, n=9-12 litters/group.

[2]One peroxidase unit = activity resulting in decomposition of one micromole of hydrogen peroxide per minute at 25°C and pH 7.0.

*Indicates statistical significance $p < 0.005$.

Serum lysozyme levels were slightly decreased in iron-deficient pups (Table 3). Wolinsky and Sherman (3) have reported that serum lysozyme is increased in iron-deficient rat pups. The discrepancies in these results may be due to the fact that pups in the present study were not as severely anemic as those in the Wolinsky and Sherman study (mean hemoglobin = 4.2 g/dl) in which dams were fed a diet containing 5 ppm iron. This severe anemia was accompanied by significant growth retardation, which was not the case in the present experiment.

Serum peroxidase is primarily myeloperoxidase (MPO), a hemoprotein which is released from neutrophils, eosinophils, and monocytes. MPO activity has been reported to be decreased (20) or unchanged (21) in iron-deficient human subjects. Baggs and Miller (22) have suggested that decreased MPO activity in iron-deficient rats could be due to the presence of fewer MPO-containing cells. The iron-deficient pups in our study had fewer circulating white blood cells (4360 $\pm$ 378 cells/mm^3) compared to controls (5296 $\pm$ 473 cells/mm^3), although this difference was not statistically significant. Since serum peroxidase activity was similar between groups, it is unlikely that the decrease in white blood cells is due to a decreased number of MPO-containing cells. However, since differential white blood cell counts were not performed, this remains unknown. As was the case with serum lysozyme, it appears that the degree of iron deficiency in the pups was not severe enough to significantly alter the activity of serum peroxidase.

The serum protein profile of dams on day 17 of lactation was relatively unaltered by dietary iron deficiency (Table 4). Total serum protein concentration and the immunoglobulins measured were similar between groups. Serum lysozyme and peroxidase concentrations were slightly lower in the iron-deficient dams; however, this decrease was not statistically significant.

TABLE 4

Serum Proteins--Dams, Day 17 of Lactation

	10 ppm Fe	250 ppm Fe
Total Protein, g/dl	5.48 $\pm$ 0.22[1]	5.45 $\pm$ 0.13
IgG, mg/dl	421 $\pm$ 43	585 $\pm$ 69
IgM, mg/dl	59 $\pm$ 8	49 $\pm$ 18
IgA, mg/dl	30 $\pm$ 4	30 $\pm$ 4
Lysozyme, µg/g protein	1.78 $\pm$ 0.20	2.30 $\pm$ 0.32
Peroxidase,[2] mU/mg protein	1.07 $\pm$ 0.22	1.48 $\pm$ 0.54

[1]Values expressed as mean $\pm$ SEM, n=9-12 dams/group. There were no statistically significant differences between groups.

[2]One peroxidase unit = activity resulting in decomposition of one micromole of hydrogen peroxide per minute at 25°C and pH 7.0.

Dietary iron deficiency resulted in decreased milk iron concentration (Table 5), which confirms previous work by Sherman et al. (23). In contrast to the differences in milk iron concentration, the milk proteins measured were similar in both groups of dams. In the human infant, these proteins are thought to provide some protection at the intestinal level against certain microorganisms (24). Our results suggest that the milk transferred to the rat pups during iron deficiency is adequate in the three immunoproteins measured: lysozyme, peroxidase, secretory IgA (sIgA).

TABLE 5

Composition of Milk and Saliva from Dams, Day 17 of Lactation

	10 ppm Fe	250 ppm Fe
MILK		
Iron, µg/ml	5.42 ± 0.95[1]	7.95 ± 0.22*
Total Protein, mg/ml	52.98 ± 1.61	53.29 ± 1.54
Lysozyme, µg/mg protein	0.60 ± 0.03	0.64 ± 0.04
Peroxidase,[2] mU/mg protein	3.67 ± 0.38	3.67 ± 0.50
sIgA, mg/dl	12.4 ± 0.5	12.5 ± 0.7
SALIVA		
Total Protein, mg/ml	3.75 ± 0.40	4.19 ± 0.29
sIgA, mg/dl	6.7 ± 0.1	6.6 ± 0.2
Lysozyme, mg/mg protein	0.9 ± 0.3	1.6 ± 0.8

[1]Values expressed as mean ± SEM, n=9-12 dams/group.

[2]One peroxidase unit = activity resulting from decomposition of one micromole of hydrogen peroxide per minute at 25°C and pH 7.0.

*Indicates statistical significance, $p < 0.05$.

There are no reports of the effects of iron deficiency on salivary proteins, which are important for both mucus structure (25) and for local host defense (26). Many proteins found in saliva are synthesized at the oral cavity mucosal surfaces. Nutritional deficiencies which alter mucosal integrity may also impair synthesis and/or secretion of some salivary proteins. Protein malnutrition, for example, is known to impair salivary flow rate, sIgA and lysozyme production and/or secretion (27). In this experiment, there was a trend toward decreased total protein and lysozyme concentrations in saliva from iron-deficient dams (Table 5), while salivary sIgA concentrations were similar in the two groups. Iron deficiency is known to alter morphology of skin and mucous membranes (28); therefore, the synthesis and/or secretion of proteins from these sites may be impaired in iron deficiency.

In conclusion, serum and secretory immunoproteins are affected only minimally in rat pups and dams with moderate iron deficiency characterized by depleted iron stores and normal growth. Although the effects of iron deficiency on the cell-mediated and humoral immunity have been studied, the secretory immune response has not been investigated in detail in either iron-deficient humans or animal models. The experiment reported here provides data for elucidation of the effects of iron deficiency on these additional components of the immune system.

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RELATIONSHIP OF SUFFICIENT AND ABUNDANT LEVEL OF MINERALS TO LIPID AND LIPOPROTEIN METABOLISM IN MALE RATS

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ABSTRACT

Recent studies have shown the effects of inidividual essential metals, or simple combinations of them, on lipid metabolism. Perturbations in blood or liver lipids due to 1% dietary cholesterol were seen in rats fed purified diets containing National Research Council (NRC) recommended levels of minerals, while rats fed chow were protected against these effects. To determine the effects of dietary minerals on lipids and lipoproteins, a study was conducted using male rats fed purified diets with two mineral mixtures, one equivalent to NRC recommendations and the other with levels similar to those present in chow. The effects of 1% dietary cholesterol in these groups were compared with those observed in chow-fed rats. The added mineral mixture affected the cholesterol distribution in the lipoprotein fractions by increasing the ratio of high density lipoprotein (HDL) to very low density + low density lipoprotein (VLDL + LDL) from 0.2 to 0.9. Moreover, it significantly reduced the total liver lipid, thus minimizing the fatty liver formation. In view of the above findings, dietary levels of essential mineral nutrients must be considered in studying the relationship of cholesterol to lipid and lipoprotein metabolism.

KEY WORDS: Rat, minerals, lipid, lipoprotein, cholesterol.

INTRODUCTION

During the last few decades hypercholesterolemia has been considered to be one of the primary risk factors in the development of atherosclerosis or ischemic heart disease. Recently, however, the distribution of cholesterol in plasma lipoproteins is considered to be a more valid parameter in the assessment of the risk than is the total plasma cholesterol. There is sufficient evidence to indicate that increased levels of plasma high-density lipoproteins (HDL) are protective in the pathogenesis of atherosclerosis (1-4), whereas elevated plasma levels of low-density lipoporteins (LDL) are associated with a high degree of risk for this disease (4-7).

Reports on the effects of a variety of dietary components on plasma cholesterol and lipoproteins are too numerous to cite. It is, however, clear that the most prominent role in hypercholesterolemia has been ascribed to the amount and type of dietary fat, in general, and to cholesterol, in particular. There are also indications that, at least in laboratory animals, other constituents of the diet may moderate or eliminate the effects of dietary fat and/or cholesterol. During nutritional studies conducted over the past few years we have repeatedly observed that animals fed purified diets showed higher serum and liver lipids than the ones fed commercial chow. Upon examination of the mineral

content of the various diets, it was evident that chow diets contained much higher levels of certain micro and macro essential minerals than the purified diets meeting the National Research Council (NRC) recommendations (8). Thus, it was hypothesized that one or more of the essential minerals, at the levels contained in commercial chow, were responsible for the protective effects against cholesterol-induced hyperlipidemia and fatty liver. This prompted us to investigate the effects of dietary cholesterol, as influenced by the total mineral content of the diet, on lipid and lipoprotein metabolism in the rat.

MATERIALS AND METHODS

Male weanling Sprague-Dawley rats with a mean body weight of 52g were randomly assigned to eight groups of seven animals. Rats were individually housed in an environmentally controlled room (9), provided with distilled demineralized water and fed ad libitum. Groups 1,2,3 and 4 were fed a purified basal diet (Table 1) containing all essential nutrients based on the recommendation of the National Academy of Science Committee on Animal Nutrition (8). Groups 1 and 2 received a dietary mineral supplement equal to that recommended by NRC (sufficient), and groups 3 and 4 received minerals approximately equivalent to those present in Purina Laboratory chow (abundant). In addition groups 5 and 6 were fed powdered Purina Laboratory chow (lab chow) to which 5% corn oil had been added (final concentration of fat 10%). Finally, groups 2,4 and 6 had 1% cholesterol added to their diet. The mineral composition of all diets is shown in Table 2. The supplemental mineral mixture was prepared by adding 312.5g potassium citrate ($K_3C_6H_5O_7 \cdot H_2O$); 160.0g potassium phosphate (K_2HPO_4); 187.5g calcium carbonate ($CaCO_3$); 62.5g, magnesium oxide (MgO); 24.125g ferric citrate ($FeC_6H_5O_7 \cdot 5H_2O$); 1.9g sodium fluoride (NaF); 31.9g zinc citrate ($Zn_3(C_6H_5O_7)_2 \cdot 2H_2O$); 0.815g cupric citrate ($Cu_2C_6H_4O_7 \cdot 2\ 1/2H_2O$); 0.04g cobalt chloride ($CoCl_2 \cdot 6H_2O$); 0.019 potassium iodide (KI); with starch as the carrier to make 1 kg. This mix was used at 4% of the diet at the expense of starch in addition to the specially prepared Bernhart and Tomarelli (10) mineral mix which also supplied 5 ppm chromium ($Cr(C_2H_3O_2)_3 \cdot H_2O$), 0.04 ppm selenium ($Na_2SeO_3$) and 1.0 ppm molybdenum ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$). The final diets were analyzed for all minerals, except fluorine, iodine, cobalt and molybdenum, by atomic absorption spectrophotometry after digesting the samples with 4:1 nitric:perchloric acids (11). The values obtained in this laboratory on several batches of Purina Laboratory chow were found to be approximately equivalent to those reported by the supplier (Ralston Purina Company, St. Louis, MO). Similarly, metal analyses of the purified diets yielded results in agreement with the values calculated from the mineral content of the salt mix and other dietary ingredients.

Rats were weighed weekly. After 169 days, they were fasted overnight and killed by exsanguination (heart puncture, after sodium pentobarbital anesthesia with 65 mg/kg body wt.). Blood was drawn from the heart for hematology, serum metals and lipid analyses. Hematocrits were obtained by centrifugation. Livers were quickly excised, blotted, weighed and stored frozen in food grade polyethylene bags until they were analyzed for lipids and metals. Tissue (approximately 1g), dried in a convection current oven at 105°-108°C to constant weight and wet ashed with 4:1 nitric:perchloric acids (12), and serum, diluted with distilled deionized water (13), were analyzed for zinc, copper and iron by atomic absorption spectrophotometry.

Table 1
Composition of the Purified Diets

	Dietary Minerals			
	Sufficient		Abundant	
Ingredients	g/kg			
Cornstarch[1]	623.5	613.5	583.5	573.5
Egg-white Solids	200	200	200	200
Corn Oil	90	90	90	90
Mineral Mix[2]	40	40	40	40
Supplemental Mineral Mix[3]	-	-	40	40
Cellulose[4]	30	30	30	30
Fat Soluble Vitamins (A,D,E)[5]	10	10	10	10
Water Soluble Vitamins	5	5	5	5
Choline Chloride	1.5	1.5	1.5	1.5
Cholesterol[7]	-	10	0	10

[1]Corn Products Co., CPC International, Inc., International Plaza, Englewood Cliffs, New Jersey, 07632. U.S.A. [2]Bernhart and Tomarelli Salt mixture (9) modified by the addition of 0.594 mg chromium acetate [$Cr(C_2H_3O_2)_3 \cdot H_2O$]; 0.4601 mg ammonium molybdate [$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$]; 0.000219 mg sodium selenite (Na_2SeO_3). This mineral mix when added at 4% of the diet supplies micro and macro minerals at levels approximately equivalent to those set forth by NRC (8). [3]This supplemental mineral mix (see text for details) was used at 4% of the diet at the expense of cornstarch. [4]Whatman CF 11, W and R. Balston, Ltd., London, England. [5]Corn oil contained (in IU/10g mixture): retinyl palmitate 30,000; vitamin D_2(calciferol) 4,000; vitamin E (d, l α-tocopheryl acetate) 100. [6]Cornstarch contained (in mg/5g mixture): thiamine hydrochloride 20; riboflavin 20; pyridoxine hydrochloride 10; calcium pantothenate 60; niacinamide 100; folic acid 0.5; menadione 2.0; biotin 1.0; i-inositol 400; cyanocobalamine (B_{12})0.02. [7]Fanchem Corp., 367 W. Erie St., Chicago, Illinois, 60610.

Table 2
Mineral Content of Sufficient, Abundant and Lab Chow Diets

	Dietary Minerals		
	Sufficient	Abundant	Lab Chow
Macro		g/kg Diet	
Calcium	9.0	12.0	12.0
Phosphorus	7.5	8.6	8.6
Potassium	2.7	10.1	10.1
Sodium[1]	3.5	3.5	4.4
Magnesium	0.6	2.1	2.1
Micro		mg/kg Diet	
Iron	37.20	198.0	198.00
Zinc	17.00	58.00	58.00
Copper	6.50	18.00	18.00
Manganese	46.00	51.00	51.00
Chromium	5.00	5.00	5.00
Selenium	0.04	0.04	-
Moylbdenum	1.00	1.00	-
Iodine	0.21	1.7	1.7
Fluorine	-	35.00	35.00
Cobalt	-	0.4	0.4

[1]Egg-White used in the preparation of purified diets contains 1.3% sodium; hence, the sodium content of purified diets is approximately equivalent to that of lab chow.

Liver total lipids were extracted according to the method of Folch et al. (14). The lipid extracts were analyzed for total lipids by gravimetric method, for cholesterol by the Lieberman-Burchard reaction (15), and for phospholipids by the method of Fiske and Subba Row (16).

Serum high density lipoprotein (HDL) was obtained by centrifugation following precipitation with sodium phosphotungstate-magnesium chloride (17). This precipation method gave HDL associated with the supernatant. Total serum cholesterol and HDL cholesterol were determined enzymatically using a commercially available kit (18). Values for cholesterol associated with very low density and low density lipoproteins (VLDL + LDL) were obtained by subtracting HDL cholesterol from total cholesterol.

STATISTICAL ANALYSIS

The response variables were checked for outliers and non-normality. Outliers were deleted and logarithmic transformations were used for serum copper, cholesterol and high density lipoprotein cholesterol, liver total lipids, and liver cholesterol. These values are reported in the tables as geometric means while the values of remaining parameters are given as arithmetic means. The data were then analyzed by two-way analysis of variance (ANOVA) and Scheffe comparisons (19). The statistical significance by ANOVA for the overall model, cholesterol and diet effects were determined. Their interactions are reported in the tables. A p value of 0.05 or less was considered as significant.

RESULTS

Periodic examination of the rats throughout the experiment did not reveal any symptoms of deficiency or toxicity in any of the groups. Growth rate as well as hematopoiesis were normal and similar in all groups. No significant changes were observed in the hematocrits and final body weights at sacrifice. However, there was an overall significant increase ($p < 0.0001$) in liver wet weight as well as specific liver weight in the cholesterol-fed groups (Table 3). Furthermore, the liver weight of chow-fed rats was greater than those of rats fed purified diets independently from the cholesterol and the mineral contents of these diets. The least increment in liver weight was evident in group 4. The liver total lipids and cholesterol, as shown in Table 4, were also elevated in the cholesterol-fed group. These increases were minimized in groups 4 and 6 indicating the protective effect of dietary minerals. Liver phospholipids, when expressed in terms of fat-free wet weight (range 35-39 mg/g), were not altered by dietary manipulations.

The data on serum cholesterol, HDL cholesterol, and the calculated values for HDL cholesterol as percent of total cholesterol, (VLDL + LDL) cholesterol, and the ratio of HDL/(VLDL + LDL) cholesterol, along with the statistical significance of effect by ANOVA, are given in Table 5. Analysis of variance of the serum cholesterol values did not show a significant effect due to dietary cholesterol. However, there was a significant effect on serum cholesterol due to the diet, to the interaction of cholesterol and diet, as well as an overall effect which indicates a disturbance in cholesterol metabolism by dietary manipulations. Among the cholesterol-fed groups the only one that showed a signifi-

Table 3
Mean Values of Body Weight and Liver Weight

Group No.[4]	Dietary Minerals: Sufficient[1] (1/2)	Abundant[2] (3/4)	Lab Chow[3] (5/6)	Statistical Significance of Effect by ANOVA: Factor	p Values
Cholesterol	Body Wt (g)[5]				
1%				Overall	NS
-	454.1	421.9	452.0	Chol.	NS
				Diet	NS
+	450.1	428.7	433.0	Chol. x Diet	NS
	Liver wet wt (g)[5]				
				Overall	< 0.0001
-	9.20^{b}_{P}	8.94^{c}_{Q}	11.06^{bc}_{R}	Chol.	< 0.0001
				Diet	< 0.0005
+	15.10^{a}_{P}	11.77^{ac}_{Q}	13.60^{c}_{R}	Chol. x Diet	< 0.004

[1]Dietary mineral levels approximately equivalent to the levels recommended by NRC(8). [2]Dietary mineral levels approximately equivalent to the levels present in laboratory chow (Data supplied by Ralston Purina Company, St. Louis, MO). [3]Five percent corn oil was added to the powdered lab chow so that the final concentration of the fat was approximately 10% (lab chow contains minimum 4.5% fat). [4]Odd numbered groups did not receive cholesterol. Even numbered groups were fed 1% cholesterol. [5]Mean values with the same matching small letter superscripts in the horizontal rows and mean values with the same matching capital letter subscripts in the verticle columns are significantly different from each other ($p < 0.05$ or less)

Table 4
Mean Values of Liver Total Lipids and Cholesterol

Group No.[4]	Dietary Minerals: Sufficient[1] (1/2)	Abundant[2] (3/4)	Lab Chow[3] (5/6)	Statistical Significance of Effect by ANOVA: Factor	P Value
Cholesterol	Liver Total Lipids (mg/g wet wt)[5]				
1%				Overall	< 0.0001
-	61_P	63_Q	60_R	Chol.	< 0.0001
				Diet	< 0.0001
+	243^{ab}_P	158^{a}_Q	148^{b}_R	Chol. x Diet	< 0.0003
	Liver Cholesterol (mg/g wet wt)[5]				
				Overall	< 0.0001
-	4.3_P	3.7_Q	$3,1_R$	Chol.	< 0.0001
				Diet	< 0.0001
+	64.8^{ab}_P	48.8^{a}_Q	42.9^{b}_R	Chol. x Diet	NS

[1-5]See Table 3 for details on foot notes

Table 5

Mean Values of Serum and Lipoprotein Cholesterol

Group No.[4]	Dietary Minerals: Sufficient[1] (1/2)	Abundant[2] (3/4)	Lab Chow[3] (5/6)	Statistical Significance of Effect by ANOVA: Factor	P Value
Cholesterol	Serum Cholesterol (mg/dl)[5]				
1%				Overall	< 0.0075
-	77.9_{P}	93.9	85.8	Chol.	NS
				Diet	< 0.0319
+	116.9^{ab}_{P}	96.1^{ac}	71.2^{bc}	Chol. x Diet	< 0.0022
	Serum HDL-Cholesterol (mg/dl)[5]				
				Overall	< 0.0001
-	50.2_{P}	53.8	48.7_{R}	Chol.	< 0.0001
				Diet	< 0.0016
+	22.4^{ab}_{P}	42.0^{a}	34.6^{b}_{R}	Chol. x Diet	< 0.0094
	HDL-Cholesterol as % Total Cholesterol[5]				
				Overall	< 0.0001
-	63.9_{P}	58.6	57.7_{R}	Chol.	< 0.0001
				Diet	< 0.0345
+	21.4^{ab}_{P}	46.0^{a}	48.2^{b}_{R}	Chol. x Diet	< 0.0012

(Continued)

Table 5 (Cont'd)

Mean Values of Serum and Lipoprotein Cholesterol

	Dietary Minerals				
	Sufficient[1] (1/2)	Abundant[2] (3/4)	Lab Chow[3] (5/6)	Statistical Significance of Effect by ANOVA Factor	P Value
Cholesterol	Serum (VLDL + LDL) Cholesterol (mg/dl)[5]				
				Overall	< 0.0001
-	31.1_p	38.0	35.2	Chol.	< 0.0004
				Diet	< 0.0144
+	91.5_p^{ab}	50.1^a	37.6^b	Chol. x Diet	< 0.0017
	Ratio of HDL/(VLDL + LDL) Cholesterol				
				Overall	< 0.0001
-	1.62_p	1.42	1.41	Chol.	< 0.0001
				Diet	< 0.0463
+	0.24_p^{ab}	0.84^a	0.92^b	Chol. x Diet	< 0.0072

[1-5]See Table 3 for details on foot notes.

Table 6

Mean Values of Serum Zinc, Copper and Iron

	Dietary Minerals			Statistical Significance of Effect of ANOVA	
Group No.[4]	Sufficient[1] (1/2)	Abundant[2] (3/4)	Lab Chow[3] (5/7)	Factor	P Value
Cholesterol	Serum Zinc (ug/dl)[5]				
				Overall	NS
-	138.0_p	131.2	131.7	Chol.	<0.022
				Diet	NS
+	123.4_p	127.1	127.6	Chol. x Diet	NS
	Serum Copper (ug/dl)[5]				
				Overall	NS
-	107.4_p	113.5	112.4	Chol.	NS
				Diet	NS
+	136.5^b_p	114.5	110.3	Chol. x Diet	<0.056
	Serum Iron (ug/dl)[5]				
				Overall	NS
-	151.3	139.8	145.3	Chol.	NS
				Diet	NS
+	146.8	159.6	245.1	Chol. x Diet	NS

[1-5] See Table 3 for details on foot notes

Table 7
Mean Values of Liver Zinc, Copper and Iron

Group No.[4]	Dietary Minerals: Sufficient[1] (1/2)	Abundant[2] (3/4)	Lab Chow[3] (5/6)	Statistical Significance of Effect by ANOVA: Factor	P Value
Cholesterol	Liver Zinc (μg/g wet wt)[5]				
1%				Overall	< 0.0001
-	33.76_{P}	33.14^{c}_{Q}	35.73^{c}_{R}	Chol.	< 0.0001
				Diet	< 0.0002
+	28.29^{b}_{P}	28.73^{c}_{Q}	31.83^{bc}_{R}	Chol. x Diet	NS
	Liver Zinc (μg/g fat free dry wt)[5]				
				Overall	< 0.0009
-	140.0_{P}	138.1_{Q}	146.1	Chol.	NS
				Diet	< 0.0005
+	152.2^{ab}_{P}	125.0^{ac}_{Q}	139.6^{bc}	Chol. x Diet	< 0.0021
	Liver Copper (μg/g wet wt)[5]				
				Overall	< 0.0418
-	4.32_{P}	4.14	4.37_{R}	Chol.	< 0.0032
				Diet	NS
+	3.63_{P}	3.99	3.84_{R}	Chol. x Diet	NS

[1-5]See table 3 for details on foot notes (Continued)

Table 7 (Cont'd)
Mean Values of Liver Zinc, Copper and Iron

Group No.[4]	Dietary Minerals: Sufficient[1] (1/2)	Abundant[2] (3/4)	Lab Chow[3] (5/6)	Statistical Significance of Effect of ANOVA Factor	P Value
Cholesterol	Liver Copper (μg/g fat free dry wt)[5]				
1%				Overall	NS
-	17.92	16.99	17.78	Chol	NS
				Diet	< 0.0431
+	19.28^{b} q	17.50	16.81^{b}	Chol. x Diet	NS
	Liver Iron (μg/g wet wt)[5]				
				Overall	< 0.0001
-	120.0^{ab}_{P}	173.7^{ab}_{P}	164.0^{b}_{R}	Chol.	< 0.0001
				Diet	< 0.0002
+	88.7^{ab}_{P}	116.7^{a}_{Q}	130.0^{b}_{Q}	Chol. x Diet	NS
	Liver Iron (μg/g fat free dry wt)[5]				
				Overall	< 0.0015
-	499.7^{b}	721.1_{Q}	669.0^{b}	Chol.	< 0.0044
				Diet	< 0.0071
+	467.8	510.5_{Q}	571.6_{R}	Chol. x Diet	NS

[1-5]See table 3 for details on foot notes

cant increase ($p < 0.001$) in serum cholesterol was group 2. Furthermore, dietary cholesterol as well as the mineral composition of the diet affected significantly the cholesterol bound to HDL. Similarly, (VLDL + LDL) cholesterol as well as the percentage of HDL cholesterol were also affected by the dietary manipulations. There was a significant effect of dietary cholesterol in the lipoprotein fractions and this was mainly contributed by the group 2. As expected, dietary cholesterol lowered the ratio of HDL/(VLD + LDL) cholesterol. This effect was more pronounced in the rats fed the diet with sufficient minerals (group 2), while it was minimized in the groups receiving the diet with abundant minerals or chow (groups 4 and 6). It is evident that the presence of abundant levels of minerals in the diet caused a favorable alteration in lipid metabolism in cholesterol-fed rats. Thus, our hypothesis has been confirmed.

Zinc, copper and iron content of serum and liver are presented in Tables 6 and 7, respectively. Analysis of variance of the data indicated no major changes in serum metals due to the dietary manipulation. The only exception is a significant ($p < 0.022$) decrease in serum zinc due to dietary cholesterol. Again, it can be seen here that this significance of effect was mainly contributed by the group fed sufficient levels of minerals (group 2).

The results on liver metals, expressed in terms of ug/g wet weight as well in terms of ug/g fat-free dry weight, are presented in Table 7. Effects of dietary cholesterol on liver zinc and copper were evident only when the concentrations of these metals were expressed on tissue wet weight basis, but not when they were based on fat-free dry weight. Thus, this effect is only secondary to the increased liver weight due to fat infiltration. The effect of cholesterol on liver iron, however, remained significant independently of the fat content of liver. Cholesterol lowered liver iron in all three groups with the lowest value occurring in rats fed diet containing sufficient level of minerals. Liver manganese values, either expressed on wet weight basis (range, 2.1 to 2.4 ug/g) or on fat-free dry weight basis (range 9.7-10.9 ug/g), were not affected by any of the dietary manipulations, since the level of dietary manganese was the same in all of the diets tested (Table 2).

DISCUSSION

The most significant findings of this study were the protective effects of the mineral content of diet on serum cholesterol and its lipoprotein distribution. It is evident from this study that dietary cholesterol decreased HDL and increased VLDL + LDL cholesterol in the rat. It is widely accepted that the partition of serum cholesterol into its lipoprotein fractions improves the predictability of the risk for cardiovascular diseases in humans (20). Thus, if HDL cholesterol is negatively correlated to the risk of coronary heart disease while the cholesterol bound to LDL and VLDL is positively associated with this risk, then the ratio HDL/(LDL + VLDL) may be a better indicator of the risk than either of these values alone. In any case, upon examination of total serum cholesterol or its lipoprotein fraction or HDL/(LDL + VLDL), it is evident that the increased mineral content of the diet offers protections against the adverse effect of dietary cholesterol. In addition to the alteration of cholesterol distribution in the lipoprotein fractions, the dietary minerals also affected fat infiltration in the liver of cholesterol-fed rats. Whether this effect is due to the altered intestinal absorption of cholesterol or to the mobilization

of cholesterol from peripheral tissues remains to be studied. In regard to the trace metal content of tissues, the data did not suggest drastic changes in the distribution of metals in the cholesterol-fed groups, possibly with the exception of iron. It is unclear at this point how the added minerals affect lipids and lipoproteins without any major changes of the trace metal content of liver and plasma. Whether the essential macro minerals are the ones responsible for moderating the effects on lipids due to dietary cholesterol, rather than the micro minerals, remains to be investigated.

We have repeatedly found that chow, in relation to purified diets, is protective against the effects of dietary cholesterol on lipid metabolism in rats (21). Others have also demonstrated the protective effects of chow diets on lipid metabolism (22) and on experimental atherosclerosis (23) in rabbits. Although there may be other dietary factors in chow influencing lipid metabolism, in this study we have definitely shown that the total mineral mixture in the diet plays a major role in the modification of the effects of dietary cholesterol on lipid levels and lipoprotein distribution.

It is felt that reduced levels of HDL cholesterol may constitute a major risk factor in the development of atherosclerosis, since HDL seems to play an important role in the mobilization of cholesterol from peripheral tissue (1). Thus, in view of the above findings, it is necessary to consider the essential mineral content of the diet in studying the relationship of dietary cholesterol and/or fat to lipid and lipoprotein metabolism. Moreover, it is important to evaluate further the role of dietary minerals, as well as that of dietary cholesterol and fat intake, in the prevention and control of cardiovascular diseases. Finally, the elevated liver weight of chow-fed rats, over that observed in the groups fed purified diets, may be attributable to the presence of hepatotoxic contaminants in the natural products which are constituents of the closed formula chow diet.

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PRESERVATION OF PLASMA DIHYDROTESTOSTERONE CONCENTRATION IN PROTEIN RESTRICTED MALE RATS: A POSSIBLE PROTECTIVE FACTOR FOR MAINTENANCE OF SPERMATOGENESIS

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ABSTRACT

Plasma 5α-dihydrotestosterone (DHT) concentration was preserved during prolonged dietary protein restriction in male rats, whereas plasma testosterone (T) concentration decreased. The ratio of plasma T:DHT was markedly lower in protein-restricted rats (0.5) than in controls (8.4). These changes in plasma androgens were accompanied by reduction in weight of seminal vesicles and ventral prostate, although testicular weight with respect to body weight was maintained. These findings suggest that during protein restriction the accessory sex organs were either insensitive to the apparently adequate levels of DHT or that DHT was not biologically available to preserve organ weight. Maintenance of plasma DHT concentration during protein restriction may be protective for spermatogenesis, since this aspect of testicular function is known to be less susceptible to the effects of undernutrition.

KEY WORDS: Androgens, protein restriction.

INTRODUCTION

The damaging effects of undernutrition on mammalian testicular function are well established (1). Impairment of Leydig cell function and hence steroidogenesis usually precedes cessation of spermatogenesis (2), resulting in a fall in total and free plasma testosterone (T) concentrations (3-5) and reduction in weight of the seminal vesicles and ventral prostate (3,4,6,7).

Reduction in T synthesis by the testis during protein and energy deprivation would seem to be related in part to reduction in serum luteinising hormone (LH) concentration (4,8,9) and reduced hypothalamic luteinising hormone releasing hormone (LHRH) content (10). However, there is evidence that undernutrition may have direct effects on the reproductive tract since the accessory sex organs of underfed bull calves showed decreased responsiveness to administered T (11). The role of 5α-reduced androgens, particularly 5α-dihydrotestosterone (DHT), in the maintenance of accessory sex organ structure and function during nutritional deprivation has not been established, although conversion of T to DHT in androgen target organs is now considered essential for preservation of their integrity (12).

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We have shown recently that both plasma T and DHT concentrations were reduced in men with undernutrition associated with chronic disease (13) although we could not be certain whether these changes in circulating androgens were related to the disease alone, to impaired nutritional status or to a combination of the two. The aim of the present study was to determine plasma T and DHT concentrations in adult male rats after prolonged protein restriction and to relate these findings to testicular and accessory sex organ size. The interrelationship between testicular steroidogenesis and seminiferous tubule function during nutritional deprivation is of particular interest since local production of DHT within the testis does appear to be important for the maintenance of spermatogenesis (14).

MATERIALS AND METHODS

Reagents and radiochemicals

Reference steroids, testosterone and 5α-dihydrotestosterone, were purchased from Sigma Chemical Company and were stored dessicated at room temperature. Standard solutions for radioimmunoassay were prepared in re-distilled ethanol and stored at -20°C. (1,2,6,7-^{3}H)-testosterone (specific activity 80-105 Ci/mmol) and (1,2,4,5,6,7-H^{3})-5α-dihydrotestosterone (specific activity 100 - 150 Ci/mmol) were supplied by the Radiochemical Centre, Amersham, UK. Solvents of analytical grade were obtained from BDH Chemicals Ltd, Poole, Dorset, U.K. and Celite analytical filter aid was manufactured by Johns-Manville, USA.

Animals and diet

Groups of 9 age and weight matched 8 to 12 week old male wistar rats, bred in the animal house at St. Bartholomew's Hospital Medical College, were exposed to a 12 hour light-dark cycle and controlled temperature 21 ± 1°C. The control group (n=9) received a 15% protein diet made up of casein, dextrin, corn starch, corn oil, agar, vitamins and salts (15,16). The experimental group (n=9) received a protein restricted diet (0.3%) which was rendered isocaloric with the control diet by proportional increases in dextrin and corn starch. All animals were fed ad libitum.

Body and organ weight

Rats were weighed daily during the 9 week experimental period. At the end of the study testes, seminal vesicles and ventral prostate were weighed, and weights were expressed in grams per 100 gram final body weight.

Plasma androgens

At the end of the experimental period animals were bled out by cardiac puncture under ether anesthesia and plasma was separated immediately and stored at -20°C, prior to assay of plasma androgens. Plasma T and DHT were determined by a previously described radioimmunoassay (13,17). Androgens were extracted from plasma with diethyl ether and T and DHT were separated by celite-propylene glycol microcolumn chromatography. This preliminary chromatographic step was necessary as the antibody used in the radioimmunoassay had 98% cross-reactivity for T and DHT, but less than 5% for all other steroids tested. Plasma androgens were determined in all samples in the same assay. Intrassay variation for these assays was less than 10%.

Statistical analysis

Differences between data from control and experimental animals were examined by the Mann-Whitney U Test.

RESULTS

Body weight

During the experimental period control animals gained 120% (93-144), i.e., median and range of their initial body weight whereas the weight matched protein-restricted experimental group lost 27% (21-44).

Testis and accessory sex organ weight

Seminal vesicle and ventral prostate weights were markedly reduced in protein-restricted rats compared to controls ($p < 0.01$) with almost no overlap of values between these groups (Table 1). Testicular weight, however, was preserved, being greater in the experimental group than in control animals when expressed in gram/100 gm body weight ($p < 0.01$).

TABLE 1.

Testis and Accessory Sex Organ Weights of Protein Restricted and Control Rats

	Control n=9 median (range)	Protein restricted n=9 median (range)	Significance P
Testis (g/100 g body weight)	0.33 (0.29 - 0.46)	0.52 (0.32 - 0.63)	< 0.01
Seminal vesicles (g/100 g body weight)	0.28 (0.23 - 0.55)	0.10 (0.05 - 0.23)	< 0.02
Ventral prostate (g/100 g body weight)	0.30 (0.24 - 0.49)	0.13 (0.03 - 0.26)	< 0.01

Plasma androgens

Plasma T concentration was significantly lower in the protein-restricted group (Table 2) compared to controls ($p < 0.025$), 8 of the 9 experimental rats (89%) having plasma DHT concentrations below the lower limit of the control range. Plasma DHT concentrations in the protein-restricted rats were all within the control range, and as a group had higher plasma DHT concentration than controls ($p < 0.05$). The ratio of T:DHT was markedly lower in protein-restricted rats (0.5) than in controls (8.4).

TABLE 2.

Plasma Androgen Concentrations in Protein Restricted and Control Rats

	Control n=9 median (range)	Protein restricted n=9 median (range)	Significance P
Testosterone (nmol/L)	9.2 (3.6 - 14.8)	1.4 (0.6 - 12.5)	< 0.025
Dihydrotestosterone (nmol/L)	1.1 (0.7 - 3.9)	3.0 (1.0 - 4.6)	< 0.05

DISCUSSION

This study demonstrates that although plasma T concentration in adult male rats falls dramatically after 9 weeks of dietary protein restriction, as has been shown previously (3,4), the concentration of plasma DHT, testosterone's potent 5α-reduced metabolite, was maintained within the control range and as a group, protein deficient rats had significantly higher plasma DHT than controls. These changes in plasma androgens were associated with marked reduction in weight of accessory sex organs, although in accordance with previous reports, testicular weight was preserved (4,18). These observations suggest that during prolonged protein restriction in the rat 5α-reduction of T is maintained, presumably at the expense of circulating testosterone levels. The findings of this study are at variance with our previous observations of plasma T and DHT in men with chronic diseases (13). This suggests that the reduction of T and DHT in these conditions may be related to a combination of disease-related and nutritional factors. Species related differences, however, cannot be excluded.

Previous studies have indicated that, compared to T, exogenously administered DHT has a similar (19-21) or increased (22,23) capacity to maintain accessory sex organ weight. Our data would suggest, therefore, that during protein restriction, the accessory sex organs are either insensitive to these apparently adequate levels of DHT as suggested by an earlier study when T was administered to underfed bull calves (11) or that DHT is biologically unavailable to these tissues. Whether the latter is related to increased plasama binding resulting in sub-normal free DHT concentration, to reduction in cytosolic or nuclear receptors, to rapid metabolism of DHT to the less potent 5α-androstanediols or to other as yet undefined factors, remains to be established.

Testicular androgens, particularly DHT are required for initiation and manitenance of spermatogenesis (14,24,25), effects which are mediated largely by sertoli cells (25). It is interesting to speculate that preservation of plasma DHT concentration during dietary protein restriction may be an important protective factor for spermatogenesis, since it is clearly more resistant than testicular steroidogenesis to the detrimental effects of undernutrition (1).

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TRACE ELEMENT REGULATION OF IMMUNITY AND INFECTION*

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ABSTRACT

Nutritional deficiency is associated with impaired immunocompetence and increased susceptibility to infection. Among other nutrients, several trace elements have been shown to regulate immune responses. Zinc deficiency results in lymphoid atrophy and decreased capacity to respond to many T-dependent antigens. Plaque forming cell response to heterologous red cells is decreased as is the function of B cells. The generation of cytotoxic spleen cells following in vivo immunization with tumor cells is reduced, whereas natural killer cell activity and antibody-dependent cell mediated cytotoxicity are largely unchanged. Iron deficiency is associated with slight reduction in rosette-forming T cells, impaired lymphocyte proliferation response to mitogens and antigens, and decreased bactericidal capacity of neutrophils. Copper deficiency results in decreased antibody-forming cell response and increased susceptibility to a variety of microorganisms. Selenium deficiency reduces T cell dependent antibody responses, particularly in association with vitamin E deficiency. Thymic hormone activity is reduced in deficiencies of zinc, copper and selenium. Excess intake of many essential trace elements also depresses immune responses. Many of the heavy metals produce impaired cell-mediated immunity. These effects of trace elements on immunity and infection may have important fundamental and clinical implications.

KEY WORDS: Trace elements, Immunocompetence, Infectious disease, Zinc, Iron, Copper, Selenium, Magnesium, Heavy metals.

* Based on a workshop held at the National Institutes of Health, Bethesda, Maryland, September 16-18, 1981. Participants: Nancy W. Alcock, William R. Beisel, Ranjit K. Chandra (Co-Chairman), Milos Chvapil, Robert A. Clark, Harvey Cohen, Gerald F. Combs, Jr., Peter R. Dallman, Delbert H. Dayton (Co-Chairman), Gabriel Fernandez, Mattie Rae Fox, Pamela F. Fraker, Janet Gregor, George H. Hass, Gerald T. Keusch, Anne K. Krey, Vinay Kumar, Orville A. Levander, Quentin N. Myrvik, Paul M. Newberne, Forrest H. Neilson, Joseph Prohaska, Merrill S. Read, Harold H. Sandstead, Maurice E. Shils, E. Richard Stiehm and Robert M. Suskind.

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INTRODUCTION

The Essential Trace Elements

As opposed to bulk elements, for example carbon and nitrogen, and macrominerals, for example sodium and calcium, trace elements occur in organisms in very low concentrations not easily estimated by earlier conventional analytical techniques and are expressed as milligrams or micrograms per kilogram of tissue; hence the term "trace" elements. Many of the trace elements are deemed "essential" because a reduced intake produces impaired function, the abnormality being prevented or corrected by physiological amounts of the specific element. Such findings should generally be observed in more than one species by several investigators. Using these criteria, the following trace elements are now accepted as essential in the growth, development and function of animals, and in many instances, of man (1,2): Arsenic, chromium, cobalt, copper, fluorine, iodine, iron, manganese, molybdenum, nickel, selenium, silicon, tin, vanadium, and zinc. The demonstration of essentiality for optimum physiologic function is easier for those elements that are needed in relatively large amounts but much more difficult to prove for those that are required in smaller concentrations. The genesis of potential adverse effects of essential trace element deficiency or excess are conceptualized in Fig. 1. We can visualize a safe

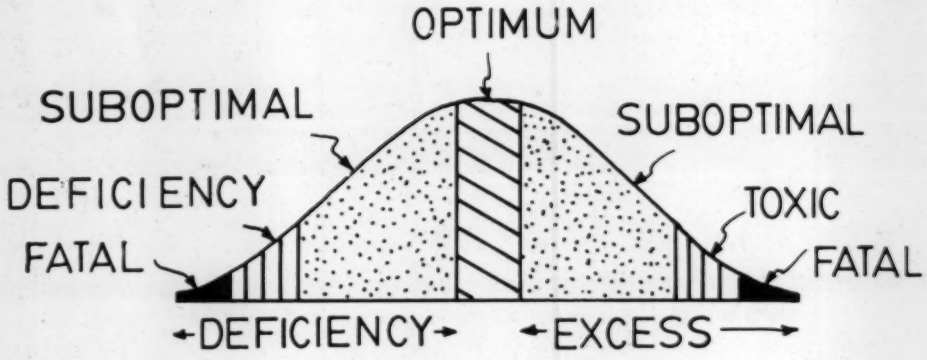

Fig. 1

Functional correlates of changes in micronutrient intake, either deficiency or excess.

range of intake and tissue concentration that permits optimal function. The essential functions are carried-out by serving as catalytic or structural components of larger molecules such as enzymes and hormones. Deficiency will result in suboptimal function, disease, and ultimately, death. Similarly, excess intake will cause toxicity that could have a fatal outcome. The shape of the biological dose-response curve varies with each trace element. The plateau of optimal function can be narrow or broad, and is maintained by a series of physiological regulatory mechanisms, such as absorption, distribution, metabolism and both specific and nonspecific excretion. These proteins, e.g. transferrin, metallothionein, may serve as regulators of trace element levels and as the means of carrying essential trace elements to specific sites where

recognition and dissociation takes place. Moreover, these proteins are normally less than completely saturated, thereby providing a buffer against excesses. Finally, the importance of interactions between trace elements should be emphasized (3). In vivo, the severity of the deficiency of one element may be aggravated by excess of another element, although, in vitro, rarely can one trace mineral be replaced by another in metaolenzyme activity.

Nutritional Modulation of Immune Responses

The concept that malnutrition enhances susceptibility to infection is based on several types of evidence: Historical, epidemiological, clinical and therapeutic. For example, the mortality rate among infants in developing countries increases progressively with worsening nutritional status, with most of the deaths being due to infection. The attack rate and duration of diarrhoeal illness are higher among those children whose weight-for-height is less than 80% of standard. The severity of infection is increased in malnourished hospitalized patients. Among children with lymphoreticular malignancy, life-threatening *Pneumocystis carinii* infection is seen mainly among those who have clinical and/or biochemical evidence of protein-energy malnutrition. These observations led to several investigations that have shown the adverse effects of malnutrition on immunocompetence (4,5). However, clinical syndromes of marasmus, kwaskiorkor and intermediate forms of undernutrition of varying severity involve deficiencies of several nutrients, each of which may play a critical role in modulation of immunity. These considerations, reviewed elsewhere, led to studies planned to look at the independent influence of single nutrients on several facets of immune response (6). The workshop summarized in this paper was convened to review the state-of-the-art of trace element regulation of immunity and infection.

ZINC

Zinc is recognized to be necessary for the growth of many species (1,7). In man, zinc deficiency is associated with growth retardation, impaired sexual development, poor healing of wounds, teratogenesis and skin rashes. The essentiality of zinc probably is based on its being an integral component of some enzymes, such as carbonic anhydrase of red blood cells, or its catalytic property for the optimum activity of other enzymes, such as DNA and RNA polymerases. Thus it is not surprosing that zinc plays a crucial role in immunocompetence (5,8,9).

In man, zinc deficiency associated with protein-energy malnutrition has been shown to contribute to impaired immunity observed in these patients. Administration of zinc resulted in an increase in the size of the thymus visualized on radiological examination of the chest. In one study, the local application of zinc sulphate ointment was associated with improved delayed cutaneous hypersensitivity response to Candida (10); however, others have not confirmed this observation (9). Occasionally, a patient receiving total parenteral nutrition may develop clinical and biochemical evidence of zinc deficiency and show impaired cell-mediated immune responses in vivo and in vitro (11). Zinc replacement corrects these abnormalities in a short period. Serum thymic factor loses much of its biological activity after passage on chelating column (8). The activity is restored after addition of zinc and to a lesser extent of some other trace elements.

Acrodermatitis enteropathica is an inherited syndrome of zinc malabsorption, characterized clinically by symmetric mucocutaneous lesions, diarrhoea, failure to thrive, and frequent severe infections with fungi, viruses and bacteria.

The thymus is small and contains reduced numbers of lymphocytes. The syndrome resembles the characteristic features of the A-47 mutant of Danish Fresian cattle. Immunologic evaluation of patients with this disease have shown impaired cell-mediated immunity as judged by cutaneous anergy and reduced lymphocyte proliferation response (12). Neutrophil chemotaxis is decreased (13). Recent data (9) suggest a selective numerical (Fig. 2) and functional deficiency of T4+ helper cells, and decreased thymic hormone activity. The immunologic changes in acrodermatitis enteropathica are corrected within a few days of providing zinc supplements.

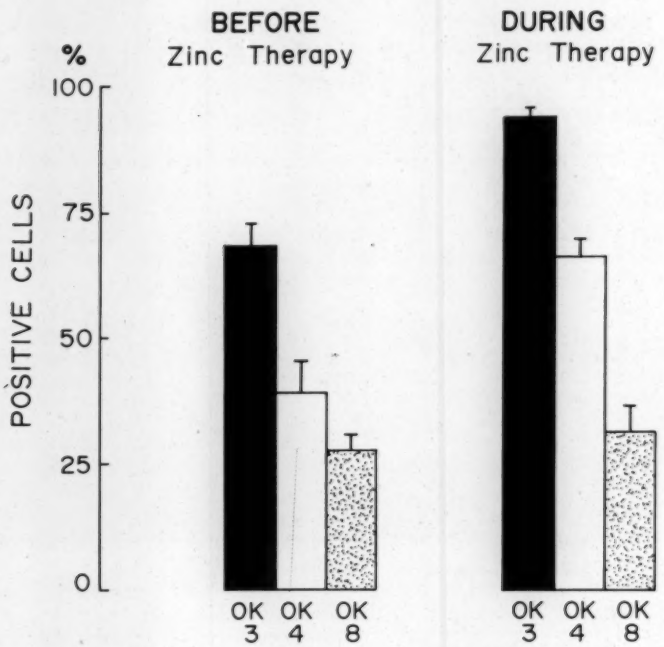

Fig. 2

T lymphocyte subsets enumerated by immunofluorescence, using monoclonal antibodies, in 5 patients with acrodermatitis enteropathica.

Much of the recent insight into the role of zinc in immunoregulation has been gained by studies in laboratory animals. Zinc deficiency in mice and rats results in rapid and pronounced atrophy of the thymus, particularly the lymphocyte-packed cortex (14, 15). Other lymphoid organs experience similar changes and the peripheral blood may show lymphopenia. Facteur thymique sérique is reduced (16, 17). Both primary and secondary antibody responses are reduced. This is established particularly for those antigens that require T cell help, such as heterologous red blood cells (14, 15, 18). Impaired T helper cell function in zinc deficient mice can be restored by providing zinc supplements (19). Nevertheless, antibody response to the T cell independent antigen, dextran is also greatly reduced. Delayed cutaneous hypersensitivity to chemical sensitizing agents is markedly decreased in zinc-deprived animals (Table 1) (9, 20). The generation of splenic cytotoxic T cells following in vivo immunization is carried out in vitro, the response is comparable with zinc replete controls

TABLE 1

Delayed hypersensitivity response to dinitrofluorobenzene in mice red various diets for 6 weeks. [From Chandra (9)]

Group	Number	Stimulation index*
Zinc deficient	7	1.41 ± 0.15
Pair-fed	7	2.09 ± 0.17
Zinc-replete ad libitum	10	2.67 ± 0.22

* Based on ratio of incorporation of ^{125}I-iododeoxyuridine into sensitized and nonsensitized skin.

(Fig. 3), suggesting the crucial role of the microenvironment of the zinc deficient organism. Natural killer cell function and antibody-dependent cell-mediated cytotoxicity are slightly altered, if at all (16). The lymphocyte proliferative response to mitogens has been reported to be subnormal or enhanced (14, 21); clearly, methodologic differences may explain such variations in

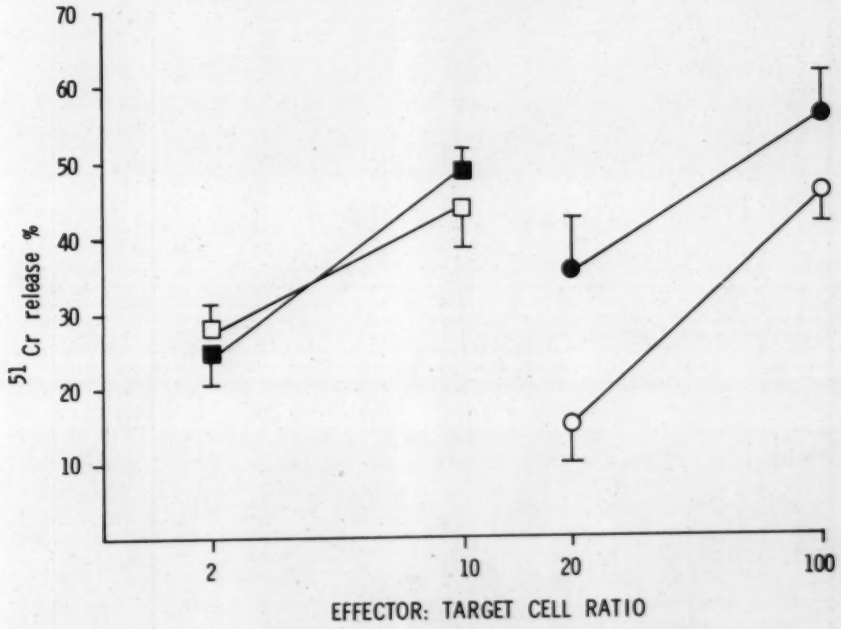

Fig. 3

Generation of cytotoxic spleen cells following in vitro (■,□) or in vivo (●,○) sensitization with tumor cells in mice. Zinc deficient animals are depicted as open symbols and controls as closed symbols.

results. If zinc deficiency is produced during the fetal or early post-natal periods, the normal ontogenetic development of the immune system is halted or slowed. On refeeding a zinc-containing diet, the deficient animals can rapidly repair immunologic deficiency developed during the period of dietary zinc restriction.

The cellular and molecular pathogenetic mechanisms underlying immunologic changes seen in zinc deficiency are complex. Zinc is essential for cell proliferation and DNA synthesis, and its deficiency will impair immune responses. In the plaque-forming cell assay, zinc was as strongly stimulating to human B cells as poke weed mitogen, purified protein derivative or diphtheria toxoid (22). Zinc is important for the optimum functioning of thymic inductive factors (8). Recent data suggest that more immature populations both of T cells and B cells accumulate in zinc-deficient animals. Elevated levels of corticosteroids may play some role in the immunologic changes seen in zinc-deprived animals (21).

The immunologic changes observed in zinc-deficient patients and laboratory animals may largely be responsible for increased susceptibility to infection. Deprived mice and rats are more vulnerable to challenge with *Listeria monocytogenes*, *Salmonella typhimurium*, *Francisella tularensis and Trypanosoma cruzi*. On the other hand, in genetically determined autoimmune disease of New Zealand Black/New Zealand White mice, zinc deficiency delays the onset and decreases the progression and severity of the hematologic and renal pathology, thereby prolonging survival (23).

Zinc excess also impairs immune response in man (9) and animals (24), both in vivo and in vitro.

IRON

Iron deficiency is the commonest nutritional problem worldwide. Individuals at increased risk include infants, particularly low birth weight infants, women in the reproductive age period, particularly pregnant and lactating women, adolescents, the elderly and the socioeconomically underprivileged. Iron is an integral part of many enzymes or serves as a co-factor for optimum function. Iron deficiency affects multiple systems including the hemopoietic, muscular, gastrointestinal, central nervous and immune systems. Both an excess and a deficiency of iron increase susceptibility to infection. In the latter, mucocutaneous candidiasis (25) and herpes simplex (26) occur frequently and respond less well to conventional therapy. In laboratory animals, dietary iron deficiency is associated with a several-fold increase in morbidity and mortality following challenge with bacterial pathogens (5, 27).

Iron deficiency impairs cell-mediated immune responses. Delayed cutaneous hypersensitivity, T cell number, lymphocyte proliferative response to mitogens and antigens, and lymphokine production are decreased to a small but measurable extent (28-33). Intracellular killing of bacteria is reduced (34). Secretory IgA antibody response is decreased (26). In contrast, there is a minimal effect of iron deficiency on immunoglobulin levels, serum antibody response, complement levels or opsonic function (6, 26).

The pathogenesis of immunologic changes in iron deficiency may include several factors. Iron is essential for the activity of the enzyme ribonucleotide reductase that is necessary for DNA synthesis and cell proliferation. It is also required for hydroxyl radical generation within phagocytes. Iron-binding lactoferrin and transferrin have bactericidal properties and they also interact with neutrophils to kill bacteria. The effects of iron deficiency on immunologic function are influenced to a significant degree by such causal factor(s) as the reduced dietary intake or blood loss that led to the iron deficiency.

Treatment with iron should be carried out cautiously in malnourished subjects who have decreased transferrin levels. In this situation, the administration of iron may activate latent bacterial infection by providing free iron for bacterial multiplication. It may also expose other deficiencies such as those of folate and zinc. Excess iron accumulation may interfere with reticuloendothelial function and compromise splenic function and macrophage activity. In the management of iron deficiency associated with protein-energy malnutrition, it is advisable to wait for 5-7 days before commencing iron replacement via the oral route which is probably without risk.

COPPER

Copper is recognized to be essential for prevention of anemia and leukopenia. It plays a crucial role in the activity of cytochrome oxidase and superoxide dismutase. It also is important for skeletal and vascular integrity due to its role in lysyl oxidase activity that is involved in the maturation of collagen and elastin. Copper-deficient animals show increased susceptibility to infection with bacterial pathogens, such as Salmonella and Listeria, but not to the same extent for coxsackie virus infection. Infants with congenital copper deficiency, Menkes disease, die in early life, usually from an infection such as pneumonia. Similarly, rare patients with acquired copper deficiency experience frequent infection. Such enhanced susceptibility to infection may be the result of neutropenia as well as impaired cellular immunity in copper deficiency. There are no definitive data on cell-mediated immune responses in human copper deficiency.

In copper-deficient laboratory animals reticuloendothelial function is impaired and granulocytes have reduced microbicidal activity. Recent studies in mice showed impaired antibody response to heterologous red blood cells (Fig. 4) (9), the extent of the deficiency correlated with reduction in ceruloplasmin (35). There was a reduction in cytotoxic activity and in lymphocyte proliferation response to concanavalin A. Thymic hormone activity is reduced (9).

SELENIUM

Selenium is essential for the activity of glutathione peroxidase which is involved in the breakdown of hydrogen peroxide and release of oxygen radicals. In selenium-deficient rats, the generation of oxygen radicals ceases with time. The effects are intensified when the cells are prestimulated by a hydrogen peroxide generating system. Selenium deficiency is associated with altered mitochondrial electron transport. Moreover, selenium is involved in the generation of prostacyclines and the chemotactic agent SHETE. Selenium deficiency, particularly in association with vitamin E deficiency, reduces antibody response measured both in terms of titer and as number of plaque-forming cells (Fig. 5) (9). Thymic hormone activity is reduced (Fig. 6) (9). Small supplements of selenium enhance antibody titers (36). Selenium in moderate amounts abrogates the immunodepression produced by methylmercury when the two chemicals are fed simultaneously to rodents. These effects are dose-dependent because higher doses of the trace element also can suppress antibody response. These interactions between selenium and the immune system may be important in the pathogenesis of the unique cardiomyopathy of man and animals encountered in the Keshan region of China where the soil and water content of selenium is extremely low. Animals challenged with viral infections were more likely to develop cardiomyopathy if they were selenium deficient. Selenium influences the vaccine-induced immunity against malaria (37).

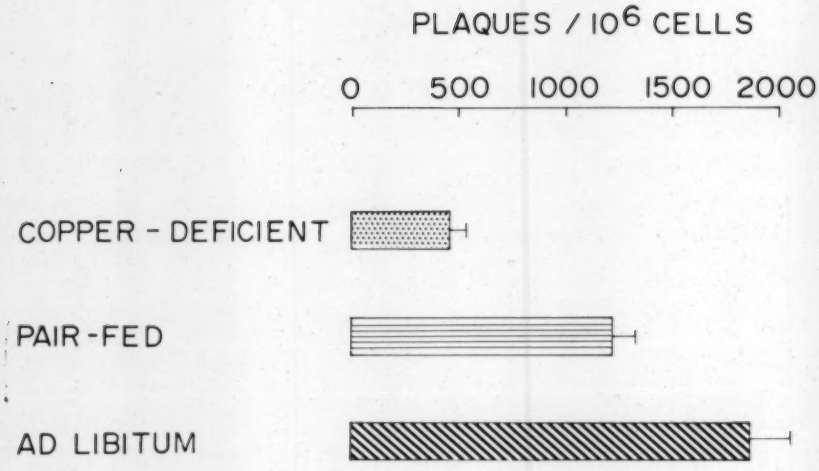

Fig. 4

Plaque-forming cell response to sheep red blood cells in spleen of copper-deficient, pair-fed and copper-replete mice fed *ad libitum*.

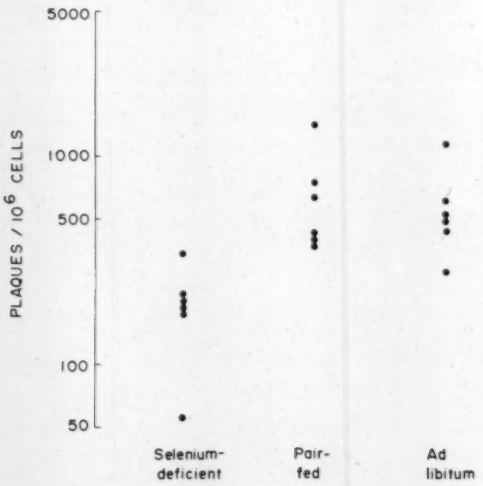

Fig. 5

Plaque-forming cell response to sheep red blood cells in spleen of selenium-deficient, pair-fed and selenium-replete controls fed *ad libitum*.

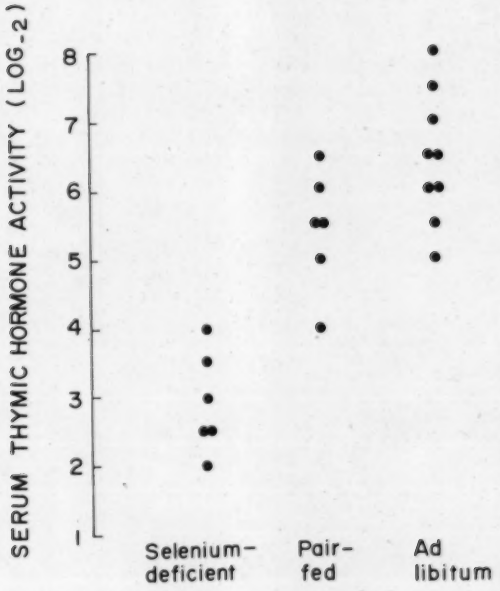

Fig. 6
Serum thymic hormone activity in the three groups of animals shown in Fig. 5.

MAGNESIUM

Magnesium is essential for normal growth of many organisms. It plays an important role in protein synthesis probably because of its effect on ribosomes. Magnesium deficiency is recognized in both man and animals. Dietary deprivation in rats results in reduced levels of serum proteins and immunoglobulins G and M (38, 39). The number of plaque-forming cells in the spleen is reduced. In a proportion of magnesium-deficient animals, thymic hyperplasia and development of malignant lymphosarcoma has been noted (40). The concurrent presence of calcium deficiency appears to promote the development of malignancy. The possibility of magnesium deficiency activating a latent virus has to be borne in mind. Alternatively, magnesium deficiency may impair cellular mechanisms for killing of tumor cells, thereby allowing spontaneously occurring malignant mutations to survive and proliferate. Data on these aspects of cell-mediated immunity in magnesium deficiency are required.

OTHER TRACE ELEMENTS

There is little information on the role of molybdenum and manganese in functioning of the immune system. Deficiencies are rare and their impact on susceptibility to infection is not known. Cobalt can influence antibody synthesis and alter susceptibility to infectious agents.

Iodine plays an important role in the microbicidal activity of polymorphonuclear leukocytes. These cells can concentrate iodide from both inorganic sources and organic compounds, such as thyroid hormones. Myeloperoxidase catalyzed oxidation of the halide by hydrogen peroxide results in the formation of intermediate molecules that are highly reactive and capable of oxidizing critical components of the target microorganism. Iodine may be covalently bound to chemical groups of bacteria and fungi. There are no systematic data on the effects of endemic iodine deficiency on host resistance. In acquired hypothyroidism secondary to Hashimoto disease, the microbicidal activity of neutrophils is decreased and improves on treatment with thyroid (4).

Data on the effects of "ultra" trace elements (arsenic, nickel, chromium, silicon, vanadium, tin, lead, cadmium, mercury, boron, lithium, etc.) on immune response are extremely limited. For many, and perhaps eventually for all of these elements, there is a continuum in which the element is essential at one level of intake and tissue concentration and toxic at another level (Fig. 1). Moreover, there are important interactions between "ultra" trace elements, with other trace elements and with other dietary factors. Absolute deficiencies of ultra trace elements are unlikely to occur in humans except among patients who have rare inborn errors of metabolism, those with metabolic alterations secondary to protein-energy malnutrition, primary disease, or stress, and those receiving total parenteral nutrition for prolonged periods.

Nickel may have an impact on the immune system via its interactions with ferric iron, zinc and copper. Nickel depresses antibody production, phagocyte function and interferon activity. Delayed hypersensitivity reactions may be slowed. It enhances infectivity of many viruses. It can induce adverse sensitivity reactions, especially skin eruptions. Arsenic can alter the production of interferon and antibodies. Cell-mediated reactions are largely unaffected. Boron may influence immunoregulatory processes. Its deficiency in chicks produces a syndrome which resembles human arthritis. Boron analogues given by intraperitoneal injections to rats and mice inhibit inflammatory arthritis. It is know to enhance cyclic AMP activity. Lithium increases mitogen-stimulated lymphocyte proliferation. It may also alter lymphocyte subsets with helper/suppressor functions. Silicon inhalation depresses alveolar macrophage activity, antibody synthesis, and T cell stimulation by concanavalin A. This inhibition can be countered in vitro by the addition of zinc which is known to stablize lysosomal membranes. Large amounts of tin may be ingested through foods packed in unlacquered cans. Tin has important interactions with the absorption and tissue retention of zinc and copper. Large amounts of tin are immunosuppressive, both antibody formation and cell-mediated responses are depressed (42). Most of the heavy metals (Lead, gold, silver, platinum, cadmium, mercury, nickel) produce hypersensitivity contact dermatitis in man and animals. Moderate exposure to these elements produces impairment of immune responses, including antibody deficiency, phagocyte dysfunction and depressed cell-mediated immunity, thereby enhancing susceptibility to certain bacterial and virus infections and some tumors.

CONCLUDING COMMENTS

The important effects of trace elements in the regulation of immune responses and modulation of susceptibility to infection are beginning to be recognized, with a growing scientific data base to support them. It is essential to bear in mind several crucial determinants that impinge on the interactions between trace elements, immunocompetence and infection. First, there are dose-related effects of trace elements on immune responses. While being essential and beneficial at one level of dietary intake and tissue concentration, the trace element may be toxic at a higher level. The range of optimum

intake may be very narrow and in many instances is not well defined. Secondly, there are crucial interactions among trace elements, with one another as well as with macro-elements and non-nutrient dietary factors, such as fiber. Thirdly, for many trace elements, absolute deficiency is rare except in patients with certain inborn errors of metabolism or those who have received total parenteral nutrition for extended periods of time. Fourthly, the inconsistent results of various studies may well be related to the mode of production of the nutrient deficiency, route of administration of the trace element, level and chronicity of deficient or excess intake and the temporal relationship between induction of deficiency and challenge with pathogen. Moreover, the outcome following infection with one type of organism does not necessarily apply to a different microorganism. Infection itself causes significant changes in the absorption, distribution and excretion of trace elements and this may produce secondary effects on immune responses. Additionally, extrapolation from in vitro studies to the in vivo situation and from animal experimentation to humans is fraught with danger. We must also define the biological relevance of immunologic changes produced in deficiency or excess of trace element intake. For example, how much reduction in antibody response or delayed hypersensitivity or number of T cells will enhance susceptibility to infection. We must separate the epiphenomena and "noise" from the clinically relevant core of truth.

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